IDENTIFICATION OF THE PHOSPHOPROTEIN BINDING SITE ON THE L POLYMERASE SUBUNIT OF NEGATIVE STRAND RNA VIRUSES

BY

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KEY TO SYMBOLS

aa	amino acid
amp	ampicillin
ATP	adenosine triphosphate
bp	base pair
BPB	bromphenol blue
CAT	chloremphenicol acetyl transferase
^{0} C	degree celsius
cm	centimeter
Ci	curie
CTP	cytidine triphosphate
DI	defective interfering particle
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EGTA	ethylene glycol-bis(aminoethyl ether)-N,N-tetraacetic acid
ETOH	ethanol
GTP	guanine triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
HSS	high salt solubilizer

hr	hour
hPIV	Human parainfluenza virus
kb	kilobase
mRNA	messenger ribonucleic acid
µCi	microcurie
µg	microgram
µl	microliter
µM	micromolar
M	molar
mCi	millicurie
mg	milligram
mm	millimeter
mM	millimolar
min	minute
m.o.i.	mutltiplicity of infection
MV	measles virus
(-)	negative sense
NEAA	Nonessential amino acids
NFDM	Nonfat dairy milk
nt	nucleotide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
^{32}P	phosphorous-32
pfu	plaque forming unit
(+)	positive sense

rpm	revolution per minute
RSV	respiratory syncytial virus
RNA	ribonucleic acid
rt	room temperature
SSPE	sclerosing panencephalitis
^{35}S	sulfur-35
s	second
SDS	Sodium dodecylsultfate
ss	single stranded
SV	Sendai virus
tRNA	transfer ribonucleic acid
US	United States
UTP	uridine triphosphate
VSV	vesicular stomatitis virus
v/v	volume per volume
w/v	weight per volume
wt	wild type
XC	xylene cyanol

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Measles and Sendai virus encode a RNA dependent RNA polymerase which is composed of the L and P proteins. In previous studies a truncation of the measles virus L protein which expressed only the amino-terminal 408 amino acids retained the ability to bind to the gstP protein. In a separate study the Sendai virus L protein retained binding when truncated to the amino-terminal 1147 amino acids. In order to identify amino acids in the L protein required for binding to the P protein, site-directed mutagenesis of the amino-terminal portion of the L proteins of both measles and Sendai virus was performed. In measles virus twelve site-directed mutants were created in the MV 408 protein and several were unable to form a complex with the gstP protein. Four of these mutants were subcloned into the full length measles virus L protein and were also unable to form a

complex with the gstP protein. These full length measles virus L proteins were then assayed for their ability to perform transcription and replication in an *in vitro* CAT assay. None of the mutants were able to give any CAT activity indicating that they were unable to form a functional polymerase complex. Nine site-directed mutants were created in the amino-terminal portion of the Sendai virus L protein and these mutant proteins were assayed for the ability to bind to the gstP protein. Of the nine mutants only the SV 516 and SV 520 proteins were able to give any complex formation with the gstP protein or form a complex with the P protein which can bind to nucleocapsids. The Sendai virus mutant L proteins were tested in various RNA synthesis experiments and only the SV 516 and SV 520 proteins were able to give any RNA synthesis. This indicates that the P and L protein must interact and form a polymerase complex before RNA synthesis can occur.

CHAPTER 1
INTRODUCTION

Background

Taxonomy

Taxonomic criteria used for the classification of viruses include genomic organization, morphology, and sequence similarity of the encoded proteins. Measles and Sendai viruses are members of the viral order Mononegevirales. Viruses of this order are characterized by a nonsegmented, single stranded, negative sense RNA genome which is tightly encapsidated by the NP or N protein. These viruses also encode for an RNA dependent RNA polymerase composed of the P and L proteins which recognizes only this encapsidated genome as a template for viral transcription and replication. The genome of these viruses follows a similar gene order with the N or NP gene first followed by the P gene of the polymerase, then the membrane associated M, F, and HN genes and finally the L gene of the polymerase. Viruses of this order also share a cytoplasmic location of the viral life cycle, transcription of subgenomic mRNAs, replication of full length RNA genomes, and a viral membrane which is derived by budding from the infected host cell.

The viral order Mononegavirales is composed of three viral families which include the Paramyxoviridae, which includes both the Sendai and measles viruses, the Rhabdoviridae, which includes vesicular stomatitis virus (VSV)and rabies virus, and the Filoviridae, which includes Ebola and Marburg viruses. Other viral families which include single stranded negative sense RNA viruses but which are segmented include the Arenaviridae, the Bunyaviridae, and the Orthomyxoviridae. The family Paramyxoviridae is composed of two subfamilies which are the Paramyxovirnae and the Pneumovirinae. The subfamily Pneumovirinae includes only one genus which is the Pneumoviruses such as respiratory syncytial virus (RSV). The subfamily Paramyxovirinae is composed of the following three genera: the Respiroviruses which include the human parainfluenza viruses type 1 and 3 (HPIV-1 and -3) and Sendai virus (SV), Rubulavirus which include HPIV-2 and -4 and the mumps virus, and Morbillivirus which includes measles virus.

Members of the viral family Paramyxoviridae share many characteristics which include the following: genome length, gene order, gene start and stop sequences, intergenic cis acting signals, and similar virus size and morphology.

Morbidity and Mortality

Viruses of the order Mononegavirales cause significant morbidity and mortality in humans even today. Vaccination

has done an excellent job of virtually eradicating many of the diseases caused by these viruses in the United States (US). Ebola, HPIV, measles, mumps, rabies, and RSV all still cause significant human morbidity and even mortality throughout the world. Outbreaks of Ebola virus occur sporadically in Africa with 70-90% of the infected people succumbing to the hemorrhagic effects of this virus. In Kikwit, Zaire, in 1995, the most recent outbreak of Ebola for which epidemiological data are available, 79% of the known infected individuals died as a result of their disease (Morbidity and Mortality Weekly Report (MMWR), 1996). HPIV can cause severe respiratory tract infection in adults and especially in children. Measles virus resulted in the death of over 1 million people throughout the world in 1998 (MMWR, 1999a). This is especially tragic considering that an effective vaccine has been available for decades. This is due to the fact that many developing countries do not have the infrastructure nor the financial resources to see that their populations are properly vaccinated. Measles virus has also been shown to be the causative agent of subacute sclerosing panencephalitis (SSPE) a secondary infection of the brain of affected individuals. This disease leads to a chronic and debilitating infection which invariably leads to death of the patient. Rabies virus persists in animal reservoirs which can lead to human infection via the bite of

an infected animal. Rabies caused 5 human deaths in the US in 2000 and an estimated $300 million dollars is spent annually on prevention and control methods (MMWR, 2000a). VSV, Newcastle disease virus, and rinderpest virus all cause significant disease in livestock with an annual cost in the billions worldwide. RSV is the predominate cause of lower respiratory tract infection in young children throughout the world (MMWR, 2000b).

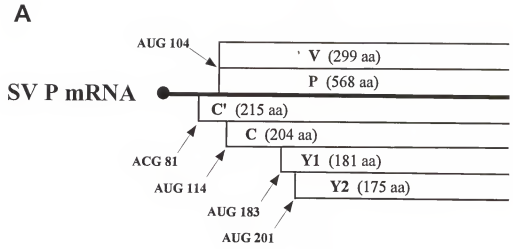
Recently two new Paramyxoviruses have been discovered, one in Brisbane, Australia, named Hendra virus and the other in Malaysia and Singapore called Nipah virus. Hendra virus was first detected as an acute respiratory infection that resulted in the deaths of 14 race horses and one horse trainer in Australia (Wang et al., 2000). Nipah virus was first detected in Malaysia as a febrile encephalitic and respiratory illness among workers with exposure to pigs. In total over 257 people are known to have been infected by the Nipah virus with 100 of the infected dying (MMWR, 1999b).

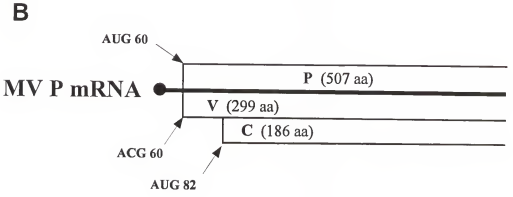
The Virion and the Viral Life Cycle

Measles and Sendai viruses are members of the viral family Paramyxoviridae. These viruses are composed of approximately 15 Kb of single stranded, nonsegmented, (-) sense RNA. Both viruses encode six mRNAs which follow the linear order of 3'-NP-P/C/V-M-F-HN-L-5' in Sendai virus and 3'-N-P/C/V-M-F-H-L-5' in measles virus (Galinski and

Wechsler, 1991). In Sendai virus the P mRNA also encodes for the a set of C proteins and the V and W proteins. These proteins are all nonstructural proteins with regulatory functions (Fig. 1A). In measles virus the P mRNA also encodes for the C and V proteins which, as in Sendai virus, have regulatory functions (Fig. 1B). The RNA genome of these viruses is always found tightly associated with the nucleocapsid protein (NP, 524 amino acids [aa] in Sendai virus or N, 525 aa in measles virus), which gives the genome its helical structure and confers resistance to nucleases such as micrococcal nuclease. This nuclease resistant characteristic is maintained even during viral transcription and replication. In Sendai virus each molecule of the NP protein is associated with exactly six nucleotides (nt) of the RNA genome. Only genomes that are multiples of six nt may be replicated efficiently (Calain and Roux, 1993). The genome of Sendai virus associates with exactly 2564 NP molecules per virion. Only this encapsidated genome can act as a template for viral transcription and replication. The virion encodes a RNA dependent RNA polymerase which consists of a homotetramer in Sendai virus of the phosphoprotein (P, 568 aa in Sendai virus and 507 aa in measles virus) and a monomer of the large protein (L, 2228 aa in Sendai virus and 2183 aa in measles virus)(Tarbouriech et al., 2000a). Within the virions the nucleocapsids associate with

Figure 1. Schematic of Sendai and measles virus P mRNA protein coding strategy. (**A**) The amino-terminus of the Sendai virus P mRNA is shown as the bold line. The translational start of the P and V proteins are shown above the mRNA, and the start sites of C', C, Y1, and Y2 are shown below. The four C start codons are in the +1 reading frame relative to the P start codon. The lengths of the proteins in amino acids are indicated in parentheses. (**B**) The amino-terminus of the measles virus P mRNA is shown as the bold line. The translational start of the P protein is shown above the mRNA, and the start sites of the C and V proteins are shown below. The V protein uses the same start site as the P protein but due to the insertion of an non-templated G residue at position 751 differs at the carboxy-terminus. The C protein is in the +1 reading frame relative to the P start codon. The lengths of the proteins in amino acids are indicated in parentheses.


A
AUG 104
V (299 aa)
SV P mRNA
P (568 aa)
C' (215 aa)
C (204 aa)
ACG 81
AUG 114
Y1 (181 aa)
Y2 (175 aa)
AUG 183
AUG 201


B
AUG 60
P (507 aa)
MV P mRNA
V (299 aa)
C (186 aa)
ACG 60
AUG 82

approximately 300 molecules of the P protein and 30 molecules of the L protein (Portner et al., 1988; Galinski and Wechsler, 1991).

Viral entry

Both Sendai and measles virus are thought to be transmitted by respiratory fluids. The surface proteins of these viruses include the hemagglutinin (HN, 576 aa in Sendai virus and H, 617 aa in measles virus) and the fusion (F, 574 aa in Sendai virus and 553 aa in measles virus) proteins. The HN protein binds to sialic acid residues on the surface receptor protein of the host cell, while in measles virus the H protein interacts with the CD 46 protein. This functions to bring the virus to the cell which allows the F protein to interact with the cell membrane. The F protein is initially synthesized in its precursor form designated F_0, which is processed by proteolytic cleavage, most likely by the endoprotease furin, into two polypeptides termed F_1 and F_2. The two polypeptides then form a covalent disulfide linkage. The F_1 polypeptide has a conserved hydrophobic domain at its amino-terminus which is exposed upon cleavage (Lamb and Kolakofsky, 1996). This hydrophobic domain is thought to interact with cholesterol within the host cell plasma membrane and plays the principal role in the fusion of the viral and host cell membranes (Eguchi et al., 2000). Once

the membranes fuse the nucleocapsid can then enter the cytoplasm of the host cell and viral transcription can begin.

Viral transcription and replication

The entire life cycle of these viruses is exclusively cytoplasmic. Upon infection of a host cell the viral RNA polymerase complex initiates transcription at the extreme 3' end of the genome. Transcription in these viruses is sequential and polar in that first a short (+) sense leader RNA is transcribed followed by the mRNAs in the order in which they appear from the 3' end of the genome. The polymerase complex fails to reinitiate ~30% of the time at each gene junction leading to a gradient of transcripts with the leader RNA the most abundant and the L protein the least abundant. The gene junctions of these viruses are highly conserved, in Sendai virus the sequence of the junction is 3'-UNAUUCUUUUU-GAA-UCCCANUUUC-5'. The first 11 nt are thought to signal the polymerase complex to terminate transcription forcing the complex to pause over the gene junction. Polyadenylation of the mRNAs is thought to occur by a stuttering mechanism over the poly U tract found within the intergenic sequence. The GAA trinucleotide is conserved at all gene junctions except the HN-L junction where it is the GGG trinucleotide. This trinucleotide is not transcribed during transcription and the sequence downstream

is thought to act as a reinitiation signal so that the next mRNA may be transcribed (Barr et al., 1997a; Barr et al., 1997b; Stillman and Whitt, 1999; Stillman and Whitt, 1998). All the mRNAs but not the leader RNA are capped, methylated, and polyadenylated (Galinski and Wechsler, 1991).

The switch from transcription to replication appears to be regulated by the accumulation of the NP or N proteins (Arnheiter et al., 1985). Once these proteins accumulate to a sufficient level there is the simultaneous synthesis and encapsidation of the nascent RNA which signals the polymerase complex to ignore the cis acting signals of the gene junctions (Vidal and Kolakofsky, 1989). This then enables the polymerase complex to generate a full length (+) sense encapsidated antigenome. These antigenomes can then be replicated into encapsidated genomes. The 3' end of the antigenome also encodes a short RNA termed minus leader which for the first 12 nt is identical to the (+) leader RNA. The remaining portion of the leader RNAs does not show a high degree of conservation. The cis acting elements necessary for polymerase initiation in replication of antigenomes reside within the first 120 nt of the genome. Promoter function for replication of genomes resides within the 3' 140 nt of the antigenome (Calain et al., 1992a; Pelet et al., 1996). Minus leader functions as a stronger

promoter for replication and as such leads to the production of many more genomes than antigenomes.

Two regions have been experimentally determined to be required for the efficient initiation of RNA synthesis. The first 31 nt are minimally necessary and sequences downstream of nt 72 in the NP untranslated region (UTR) are also essential (Pelet et al., 1996). In *Paramyxoviruses* there is a semi-conserved region termed the BB box located between nt 79-96 which may play a role in the initiation of RNA synthesis (Tapparel et al., 1998). In the replication of *Paramyxoviruses* the rule of six may also play an important role (Egelman et al., 1989; Calain and Roux, 1993) but this does not appear to be true for all *Paramyxoviruses*. The conserved 12 nt of the leader RNAs are important for initiation of RNA synthesis in that they act as the first two NP-RNA hexamers. It is thought that the region between nt 47-67 is important for the proper phasing of the NP-RNA hexamers. The phasing of the NP-RNA hexamers is thought to be a third element in the promoter function for replication (Kolakofsky et al., 1998). The BB box contains three NP-RNA hexamers and they are positioned exactly 13 NP-RNA hexamers from the 3' end of the genome. This distance is significant in that it corresponds to one turn of the helical genome which in effect puts the two putative promoter regions in close proximity on the same side of the genome (Pelet et

al., 1996). Similar structures of these cis-acting sequences have also been discovered in both Simian virus 5 (SV-5) and HPIV-3. In these viruses the 3' end of the nucleocapsid RNA as well as the sequence one helical turn away were found to be essential for viral RNA synthesis (Murphy et al., 1998; Hoffman and Banerjee, 2000). It is thought that these cis-acting signals on one face of the nucleocapsid act as a polymerase binding site.

The L and P proteins must be co-expressed in order to form a functional polymerase complex (Portner et al., 1988; Horikami and Moyer, 1995; Horikami et al., 1992). For both transcription and replication, the model of polymerase function is that the P protein functions to bind the polymerase complex to the encapsidated genome. The L protein is thought to carry out all enzymatic functions such as initiation, elongation, termination, polyadenylation, and capping and methylation of the 5' end of the mRNAs (Gotoh et al., 1989; Horikami and Moyer, 1995; Tarbouriech et al., 2000a). It is known that the SV L protein binds between the aa 412-445 of the SV P protein but prior to this work it was not known where on the L protein the P protein binds.

Viral morphogenesis

Both Sendai and measles virus have a lipid bilayer which is derived from the infected host cell. This viral membrane contains the two membrane bound proteins, the F

and the H(N) proteins. Both proteins have signal sequences that target them to the ER during their translation and as a result they become membrane bound (Lamb and Kolakofsky, 1996). The third structural protein found in both viruses is the matrix protein (M, 348 aa in Sendai virus and 335 aa in measles virus). This protein functions in morphogenesis of the progeny viruses. This protein has been shown to associate with the cytoplasmic tails of the F and HN proteins and with the NP protein. It is thought that the M protein functions as a bridge between the host membrane and the nucleocapsids which can then aid in the morphogenesis of the virions (Lamb and Kolakofsky, 1996).

Reverse Genetics

The problem of working with (-) strand RNA viruses is the lack of molecular biological techniques for working directly with the RNA genome. Until recently creation of site directed mutants in each of the individual proteins could be studied in the context of a viral infection was impossible. The life cycle of these viruses has no DNA intermediate with which standard molecular biological technique could be used so a reverse genetics technique has been developed. This technique allows the rescue of viruses from cDNA copies of the full length genome utilizing plasmids encoding the proteins important for transcription and replication, namely the N or NP, P and L proteins.

Viruses that have been rescued using this technique include hPIV-3 (De and Banerjee, 1993), canine distemper virus (Gassen et al., 2000), measles (Radecke et al., 1995), mumps (Clarke et al., 2000), rabies (Schnell et al., 1994), RSV (Collins et al., 1995), rinderpest (Baron and Barrett, 1997), Sendai (Garcin et al., 1995), SV-5 (He et al., 1997) and VSV (Lawson et al., 1995). Plasmids which express the full length genome plus separate plasmids which encode the N or NP, P and L proteins are transfected into cultured cells. These plasmids are all under the T7 polymerase promoter and as such it is essential that the cells express the T7 RNA polymerase. Mammalian cells are infected with VVT7, a vaccinia virus recombinant engineered to express T7 RNA polymerase from an early/late promoter. The second is to use cells transfected with the T7 RNA polymerase such that the cells endogenously express the T7 RNA polymerase. The plasmid that encodes the full length genome produces a RNA copy of the genome which is positive or antigenomic sense. This is so that the mRNAs encoding the viral proteins do not anneal with the (+) sense anti-genomic RNA. The genome created by this technique must have authentic viral ends to ensure that both viral transcription and replication can occur. The 5' end of the DNA includes the hepatitis delta virus ribozyme which cleaves itself out of the transcript generating an authentic 3' end. The transcript must then be

nonspecifically encapsidated by the NP or N protein in order to generate a nucleocapsid which can then act as a template for both transcription and replication by the viral polymerase complex. The remaining viral proteins are then synthesized by this rescued genome and the virus can complete its life cycle. The end result is the rescue of a virus which should incorporate any changes made to the cDNA copy of the genome.

Defective Interfering Particles

Defective interfering (DI) particles contain subgenomic length RNAs that require coinfection by a wt virus that is able to provide helper functions in trans. These helper functions must include any genes missing in the DI genome. These particles are defective because alone they are unable to complete the viral life cycle because they are deficient in some required function. They are interfering because they inhibit the replication of wt virus. Since DI particles have genomes smaller than their full length counterparts they are able to replicate much quicker and as such tend to out compete their parents with a larger genome. There are at least two ways that DI particles can be generated in these viruses. First the polymerase during replication may skip large portions of the genome and then reinitiate near the 5' end. This would generate a DI with

authentic 5' and 3' ends but would have a potentially large internal deletion (Re and Kingsbury, 1986).

The other way to generate a DI is to create a copy back DI. This happens when the polymerase complex initiates replication at the 3' end of an antigenome; replication proceeds but then the polymerase disassociates from the template and the polymerase then continues replication on its own nascent RNA. This generates a copy back DI with inverted repeats of the 5' end of the genome. The end result is a DI that has a strong replication promoter on each end (Calain et al., 1992b). In VSV, but not with other viruses, it has been shown that complementarity between the 3' and 5' ends corresponds with increased replication efficiency. The copy back DI has the strong replication promoter at each end. The copy back DI also has increased complementarity because each end is a copy of the other and this leads to a significant replicative advantage as compared to deletion DIs and the wt virus (Wertz et al., 1994).

A cDNA copy of a Sendai copy back DI termed DI-H can be used in a minigenome rescue system. A useful technique for the study of Sendai virus *in vivo* replication employs an infection/transfection method whereby cultured cells are infected by VVT7 and then transfected with the plasmids encoding for the N, P and L viral proteins and the DI-H copy

back DI which are all under the control of the T7 RNA polymerase promoter. As with full length rescue described in the previous section the plasmid encoding the DI-H generates a positive sense minigenome. The minigenome must then be nonspecifically encapsidated by the NP protein and then the nucleocapsid becomes a template for replication. This technique is termed *in vivo* replication (Curran and Kolakofsky, 1991). Another technique to study replication *in vitro* instead of in the cell is to infect cells with VVT7 and transfect them with the plasmid encoding for the NP, P and L proteins and then prepare cell extracts. These extracts expressing the viral proteins can then be used to support *in vitro* replication or transcription by the addition of DI-H or wt virus polymerase-free nucleocapsids, respectively (Carlsen et al., 1985; Horikami et al., 1992; Wertz et al., 1994).

Viral Proteins Required for RNA Synthesis

The NP protein

The Sendai virus NP protein is endoded by the first gene in the linear order of the genome. After the leader RNA, the NP mRNA is the most abundant viral transcript present in an infected cell. The NP protein is a multi-functional protein that must interact with the viral genome to form the nucleocapsids and it also must interact with other viral proteins to complete the viral life cycle.

Formation of the nucleocapsid is crucial in order that the single strand RNA genome is protected and is also critical so that the polymerase complex can recognize the viral genome. It is well known that the polymerase complex interacts with the RNA genome via the P protein of the polymerase complex and the NP protein of the nucleocapsid (Buchholz et al., 1994). This encapsidation by the NP protein specifically protects the genome from nucleases even during transcription and replication. In order to encapsidate the genome so tightly the NP protein must interact with neighboring NP proteins. This forms a continuous multimer of NP proteins which can then tightly associate with the RNA genome (Baker and Moyer, 1988; Buchholz et al., 1993). The NP protein when expressed alone self-aggregates into nucleocapsid like particles due to its ability to form multimers around cellular RNAs (Buchholz et al., 1993). In order to prevent the NP protein from aggregating the NP protein binds to the P protein which helps to keeps it soluble (Horikami et al., 1992). This P-NP_0 heterodimer is the substrate the viral polymerase uses to encapsidate the nascent replicating RNA (Horikami et al., 1992). Finally the NP protein of the nucleocapsid is also able to interact with the M protein. This interaction is thought to bring the encapsidated genomes of the virus to

the plasma membrane of the infected cell so that the virus may bud from the infected cell (Stricker et al., 1994).

The NP protein is divided into two functional domains, the amino terminal 80% of the protein is important for NP-NP and the NP-RNA interactions (Parks et al., 1992). This domain is highly conserved among the Paramyxoviruses. Upon treatment of the nucleocapsids with trypsin a 48 kilodalton (kd) fragment which corresponds to the amino-terminal 80% of the NP protein remains associated with the RNA (Heggeness et al., 1981). These protease treated nucleocapsids retain their nuclease resistance and upon examination by electron microscopy they appear as authentic nucleocapsid structures. The remaining carboxy-terminal 20% is important for NP-polymerase interactions. This carboxy-terminal domain displays little homology among the members of the Paramyxoviruses. This is the portion of the NP protein that is lost when the nucleocapsids are treated with trypsin. This region of the NP protein contains a majority of the phosphorylation sites of the protein and it also contains the antigenic portion of the protein (Hsu and Kingsbury, 1982; Fisher, 1990; Ryan et al., 1993). Antibodies to this region also disrupt the ability of the P protein to bind to the nucleocapsids (Ryan et al., 1993). As a result of these observations it is thought that this carboxy-terminal

portion of the protein is on the surface of the nucleocapsids.

The Sendai virus NP protein has been subjected to extensive deletion and mutagenesis analysis across its length. The use of deletion mutants across the amino-terminal 400 aa showed that this region of the protein is important for NP-NP and P-NP_0 interactions, and for *in vitro* replication (Buchholz et al., 1993; Curran et al., 1993). It was also shown by deletion analysis that the carboxy-terminal portion of the protein was not required for nucleocapsid assembly, but was necessary for template function and recognition by the polymerase complex which is required for replication. A deletion of aa 426-524 was unable to bind to the P protein (Curran et al., 1993), and while aa 440-524 were required for P protein binding a deletion of aa 400-439 had no effect on P protein binding (Buchholz et al., 1993). The polymerase complex binds to nucleocapsids via the P protein and any NP-RNA complex that is unable to bind to the P protein would be defective in all RNA synthesis because it would be unable to serve as a template. Site directed mutants within a highly conserved and charged region of the NP protein between aa 107-129 were able to form the P-NP_0, NP-NP, and the NP-RNA interactions but were unable to support *in vivo* replication (Myers and Moyer, 1997). This indicates that this region of the NP

protein as well as the carboxy-terminal portion of the protein are also necessary for the nucleocapsids to function as a template for replication. Maltose binding protein (MBP) fusion mutants between the central conserved region (CCR) (aa 258-357) and the amino-terminal 255 aa could each oligomerize indicating that both regions are important for the NP-NP interaction (Myers et al., 1997). In line with this the point mutant at P324A was unable to self assemble indicating that this site is important for the NP-NP protein interaction. Other point mutations at T260A which is with in the CCR and F362A which is nearby resulted in mutant proteins which gave aberrant NP-NP interactions. Upon examination by electron microscopy these nucleocapsids lacked the usual helical structure and encapsidated RNA indicating that these two positions are important for the NP-RNA interaction (Myers et al., 1997; Myers et al., 1999). Other point mutations at L299, I300, I313, K370, and D371 were all able to form NP-NP and P-NP_0 protein interaction but were all inactive in viral replication (Myers et al., 1999; Myers et al., 1997). This suggests that these point mutants were all deficient in the NP-RNA interaction and thus were unable to encapsidate the nascent RNA. These data together indicated that the CCR contains aa important for the NP-NP and P-NP_0 protein interactions.

The measles virus N protein has an overall structural similarity with the SV NP protein. The measles virus N protein is also the first transcript after the leader RNA and is the most abundant viral mRNA present in infected cells. The amino-terminal portion of the N protein is also important for N-N and N-RNA interactions and the carboxy-terminal portion is important for the P-N_0 interaction. Deletion mutants within the amino-terminal 492 aa abolished or severely inhibited the ability of the N protein to form the N-N or N-RNA interactions (Bankamp et al., 1996). The exact region of the N protein required for the N-RNA interaction in measles is unknown but sequence alignments with NP of other viruses have revealed a region of high conservation near the middle of the protein. This region termed the central conserved region (CCR) is presumably important for one of the N protein interactions and could play a role in the nucleocapsid formation as in Sendai virus (Miyahara et al., 1992).

The P protein of measles and Sendai viruses

The P transcript

The Sendai virus P gene is a unique in that it codes for 6 proteins in addition to the P protein (Curran and Kolakofsky, 1989) (Fig. 1A). The C proteins are a group of four nonstructural proteins called C′ (215 aa), C(204 aa), Y1 (181 aa), and the Y2 (175 aa) proteins. These proteins

are expressed from alternate start sites (nt 81, 114, 183, 201, for C', C, Y1, and Y2, respectively) in the +1 reading frame as compared to the P protein (nt 104) (Gupta and Patwardhan, 1988; Giorgi et al., 1983; Curran and Kolakofsky, 1988). The C' protein uses an alternate start site with the sequence ACG instead of the traditional AUG (Curran and Kolakofsky, 1988). Translation of the C', P, and the C open reading frames appears to be by a ribosome scanning method. This is thought to be possible because each of the start sites is in an increasingly stronger Kozak sequence context with the optimal Kozak sequence of PuNNAUGG (Kozak, 1986). Interestingly if the ACG sequence of the C' start site is changed to an AUG which places this start site in the optimal Kozak sequence then translation of the downstream P and C proteins is abolished (Curran and Kolakofsky, 1989). In this case the Y1 and Y2 proteins are still translated apparently by a cap-dependent but scanning-independent process (Curran and Kolakofsky, 1989). This is thought to occur by a ribosomal shunt where the ribosome initiates at the cap structure, begins scanning for a start site, but then is translocated to the start site of either Y1 or Y2 by a ribosome donor and acceptor site within the mRNA sequence (Latorre et al., 1998). It is likely that the structure of the P mRNA helps to facilitate this ribosomal shunt mechanism (Gupta and Ono, 1997).

The V protein of Sendai virus is created when an untemplated G residue is incorporated at a specific site during transcription of the P mRNA. This process has been termed RNA editing. This causes a frame shift at aa 213 during translation of the mRNA that results in the V protein with a different carboxy-terminal 68 aa as compared with the P protein (Vidal et al., 1990; Curran et al., 1991). The W transcript of Sendai virus is created by the addition of two nontemplated G residues in the P mRNA which results during translation in a truncation of the W protein shortly after the editing site.

The measles virus P gene encodes 3 proteins including the P, V, and C proteins. The V mRNA uses the same transcription start site as the P gene but an nontemplated G residue is incorporated at nt 751 of its mRNA. This gives a frameshift where the translated V protein is therefore identical to the P protein for the first 231 amino-terminal aa, then the last 68 aa are translated from the V reading frame (Tober et al., 1998). This carboxy-terminal 68 aa is rich in cysteine, as is the Sendai V protein, which is thought to form a zinc finger domain which is capable of binding to zinc with high specificity (Liston and Briedis, 1994). The V protein is not associated with intracellular viral particles and recently has been shown to be

dispensable for the MV infection cycle in cell culture (Schneider et al., 1997).

The measles virus C protein is encoded by a separate start site 22 nt downstream of the P and V proteins start site on the P mRNA. The C protein is in the +1 reading frame as compared to the P protein. Translation of the C protein is due to ribosomal scanning due to the fact that the C start site is in a stronger Kozak sequence context than the P and V start site. As with the V protein, the C protein of measles virus is dispensable for viral propagation in cell culture (Radecke and Billeter, 1996), but it appears to be required for replication in peripheral blood cells (Escoffier et al., 1999).

The P protein

The P proteins of measles and Sendai viruses are multi-functional proteins. Recently the structure of the oligomerization domain of the Sendai virus P protein was solved by high resolution X-ray crystallography revealing a homotetrameric coiled coil structure (Tarbouriech et al., 2000a). Together with the L protein the P protein forms the RNA dependent RNA polymerase. These two proteins must be co-expressed in order to form a functional polymerase complex because the P protein is required for the correct folding and stabilization of the L protein (Horikami et al., 1992). The L-P polymerase complex is stable for 15 hr while

the L protein without coexpression of the P protein is stable for only 1.2 hr (Horikami et al., 1997). The P protein also complexes with the NP protein which keeps the NP protein soluble as described above. These P-NP_0 complexes serve as the substrate for encapsidation of the nascent RNA during replication (Horikami et al., 1992; Curran et al., 1995a). The P protein also has a supplemental role in replication and transcription where oligomers of the P protein interact, independently of the L protein, with the nucleocapsids (Curran, 1996; Bowman et al., 1999). This interaction may play a role in removing or altering the NP-RNA complex so that the polymerase has access to the genomic RNA during transcription and replication.

The P protein forms many protein-protein interactions in connection with its many functions. The regions important for these protein-protein interactions have been delineated by deletion analysis. The aa important for the P protein to bind to the L protein are between aa 412-445 in Sendai virus (Smallwood et al., 1994; Curran et al., 1994). The regions on the P protein important for binding to the NP protein of the encapsidated genome are between the aa 345-422 and 479-568. These regions are non-contiguous and they flank the region important for L protein binding to the P protein (Ryan and Kingsbury, 1988; Ryan and Portner, 1990;

Ryan et al., 1991). The aa important for formation of the P-NP_0 complex are also non-contiguous domains between aa 33-41 and 479-568 (Curran et al., 1995a). A P protein with only the aa 78-316 also retained binding to the NP protein indicating that these aa also play a role in the formation of the P-NP_0 complex (Horikami et al., 1996).

The P oligomerization domain resides between aa 344-411 and it was thought initially that the P protein formed a homotrimer (Curran et al., 1995b) but it is now known to form the homotetrameric coiled coil (Tarbouriech et al., 2000a). Other regions of the P protein are also important for the transcription and replication functions of the polymerase complex. The region between aa 1-77 is important for genome encapsidation during replication while the region between aa 78-114 is required for viral transcription (Curran et al., 1994).

The P proteins of viruses of the order Mononegavirales are phosphorylated at a number of sites. For Sendai virus P protein phosphorylation at position S249 accounts for 80% of the phosphate content (Byrappa et al., 1996). When the mutant S249A was created in the SV P protein it was discovered that this did not reduce the amount of P protein phosphorylation. In fact a S249A mutant virus grew to wt levels in tissue culture as well as in the mouse lung. This was due to increased phosphorylation at alternate sites

within the P protein. The dominate alternate sites in Sendai virus P protein are at locations S419 and S426, but even after these alternate phosphorylation site were eliminated the mutant Sendai virus P protein was still able to perform *in vitro* replication and transcription (Kato et al., 1997). This indicates that in Sendai virus, P phosphorylation is not required for transcription, replication, or protein oligomerization. In contrast, the phosphorylation of the VSV P protein occurs at three main sites, namely S60, T62, and S64 (Chen et al., 1997), while the phosphorylation positions in hPIV-1 are at two main sites, namely S120 and S184 (Byrappa and Gupta, 1999). Phosphorylation at these sites is required for the appropriate folding and oligermerization of both the VSV and hPIV-1 P proteins and is also essential for transcription. Thus phosphorylation of the P protein is required in some viruses like VSV, RSV, and hPIV, but not in others like Sendai virus (Hu et al., 1999).

The C proteins

The Sendai C proteins are a set of four proteins termed C', C, Y1, and Y2. These proteins are present at extremely low amounts in virions but are present at relatively high amounts in the cytoplasm of infected cells as determined by immunofluorescence (Yamada et al., 1990). The C protein is present in the highest amounts of the four proteins with

approximately equimolar amounts as compared with the P protein. This work is only concerned with the C protein and its interaction with the polymerase complex. It has been shown that the C protein can bind to nucleocapsids associated with the polymerase complex but not with polymerase-free nucleocapsids. The C protein is able to bind to the L protein component of the polymerase complex and it is through this interaction that the C protein is able to regulate RNA synthesis (Horikami et al., 1997). The C binding site on L is not currently known but it is distinct from the P protein binding site.

When the C open reading frame was removed from a plasmid expressing the P and C proteins *in vitro* transcription increased. When the C protein was added back by transfection of a plasmid encoding only the C protein the increased transcription was eliminated (Curran et al., 1992). Thus the C proteins down regulate transcription. Initial results indicated that replication of the copy back DI (DI-H) was not affected by the presence or absence of the C protein, so it was thought that the C protein inhibited transcription but not replication (Curran et al., 1992). Recently it has been shown that the C protein does have an inhibitory effect on the replication of internally deleted DI particles, wt template replication *in vivo*, and *in vitro* replication of DI-H (Cadd et al., 1996). The difference

between the two types of replication is in the viral termini and as a result it is thought that the ability of the C protein to inhibit RNA synthesis is promoter specific. It has been shown by others in our laboratory that the C proteins acts to inhibit viral replication and transcription and that this inhibition requires that the C proteins be able to bind to the L protein (Horikami et al., 1997).

It has also been shown that the C protein plays a role in viral pathogenesis in that a point mutant F170S completely prevented the inhibition of mRNA synthesis by the C protein. Viruses with this mutation do not produce a lethal infection in an animal model (Cadd et al., 1996). Recently several C protein knockout viruses have been constructed using reverse genetics. The viruses were difficult to rescue and indeed grew poorly but were still viable indicating that the C proteins are not absolutely required for viral replication and transcription (Kurotani et al., 1998). Other functions attributed to the C protein include inhibition of the α/β-interferon response of infected cells (Gotoh et al., 1999; Garcin et al., 1999) and more recently as a contributor to viral assembly (Hasan et al., 2000).

The L protein

Alignment of the L proteins of the viruses of the order Mononegevirales shows six domains of relatively high

conservation (Sidhu et al., 1993; Poch et al., 1990) (Fig. 2). Domain II has a motif of alternating basic and hydrophobic residues which may form an amphipathic α-helix with RNA binding activity and it is thought that this domain mediates interaction between the polymerase and the RNA genome. Domain III contain the GDNQ sequence which is thought to code for the RNA polymerization activity of the polymerase. When this sequence was mutated by site directed mutagenesis in VSV and rabies viruses, it was found that the conserved GDN sequence was required for polymerase function (Schnell and Conzelmann, 1995; Sleat and Banerjee, 1993). Domain VI has a purine binding motif which may bind ATP. The other domains of the L protein have no recognizable motifs and their functions are currently unknown.

Together with the P protein the L protein is a component of the RNA dependent RNA polymerase (Horikami et al., 1992; Banerjee and Barik, 1992; Horikami et al., 1992; Banerjee and Barik, 1992). This P binding stabilizes the L protein probably by helping the L protein to fold correctly (Horikami et al., 1992; Horikami et al., 1997). The L protein is thought to code for all catalytic functions of RNA synthesis such as: RNA polymerization, ribonucleotide binding, mRNA capping, methylation of the cap, and polyadenylation (Hunt and Hutchinson, 1993; Hercyk et al., 1988). A mutant F1488S in the VSV L protein conferred an

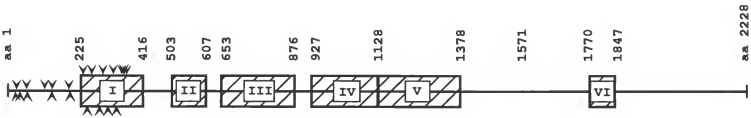


Fig. 2. Sendai virus L protein schematic. Conserved domains I-VI (Poch et al., 1990) are shown as shaded boxes with their amino acid boundaries indicated above. The ▲ below the line indicates the positions of mutations constructed in Sendai virus L as described in the text. The ▲ above the line indicates the relative positions of mutations constructed in MV 408 as described in the text.

aberrant polyadenylation phenotype giving long poly(A) tails (Hunt and Hutchinson, 1993). When this mutant was reconstructed by site directed mutagenesis within the SV L protein, it resulted in a defect of transcription but did not recreate the polyadenylation phenotype (Horikami and Moyer, 1995). The L protein has also been shown to have a kinase activity which may play a role in phosphorylating the NP and P proteins (Einberger et al., 1990; Hammond et al., 1992). It is currently unknown where the various catalytic activities of the L protein reside.

In order to study the P and L protein interactions, truncations of Sendai and measles L proteins have been analyzed for their ability to bind to the P protein (Chandrika et al., 1995a). In Sendai virus the L protein was truncated to the amino terminal 1147 aa and still retained the ability to bind to the Sendai P protein. Any further truncations of the Sendai virus L protein lost the ability to bind the P protein (Chandrika et al., 1995a). Similar studies performed with the SV-5 L protein mapped the P protein binding site to the amino-terminal 1247 aa (Parks, 1994a). In measles, however, a truncation to the amino-terminal 408 aa retained measles P protein binding (Horikami et al., 1994). Site-directed mutants were created in domain I of the Sendai L protein and were assayed for their ability to bind the Sendai P protein. Polymerase complex formation

was assayed using a glutathione-s-transferase and Sendai P fusion protein (gstP) (Chandrika et al., 1995a). This SV gstP protein is known to form a complex with wt Sendai L which can then be pulled down using glutathione conjugated Sepharose beads. This assay was used to see if the mutant L proteins were also able to form this complex. The result was that only one of the nine mutants, specifically S368R, lost binding to gstP protein (Chandrika et al., 1995a). This particular mutant was interesting because it replaced the Sendai aa with the corresponding measles virus aa at that position.

It has been shown that measles P protein does not bind Sendai L protein nor does the Sendai P protein bind to measles L protein (Chandrika et al., 1995a). Portions of the measles virus domain I were subcloned into Sendai virus domain I to determine if this Sendai L hybrid could then bind to the measles virus P protein, but it was unable to bind. This leads to the conclusion that it is the differences between the two L proteins and not the similarities that confer P protein binding specificity (Chandrika et al., 1995a). Recently the L protein of VSV has been shown to associate with cellular elongation factor (EF-1) and this host protein is required for polymerase activity (Das et al., 1998). It remains to be seen if

Sendai or measles viruses require any host factor for polymerase activity.

Our laboratory has created a number of site directed mutants in each of the six domains of the Sendai virus L protein. In domain I only one mutation gave an L protein unable to bind to the P protein and several of the other mutations uncoupled transcription and replication (Chandrika et al., 1995a). Domain II is thought to have a RNA binding motif which is important for interaction between the polymerase complex and the viral genome (Muller et al., 1994). Mutants in domain II are defective in RNA synthesis with five of seven mutants unable to synthesize any RNA at all (Smallwood et al., 1999). The two remaining mutants have different phenotypes in which one gave transcription and some replication in vitro but not in vivo and the other mutant could transcribe but not replicate thus uncoupling transcription and replication. Mutants in domains IV and VI gave a spectrum of phenotypes ranging from near wt levels of RNA synthesis to the complete elimination of all RNA synthesis (Feller et al. 2000). Mutants in domain V also gave a spectrum of phenotypes ranging from mutants which could transcribe leader RNA well but not mRNA, to mutants that could generate more mRNA than the leader RNA (Cortese et al., 2000). Mutants in domain V also uncoupled transcription from replication with some able to do

replication *in vivo*, but not *in vitro*. All the mutants generated in the domains of the SV L protein, with the noted exception of one in domain I, were able to form a polymerase complex and to bind to the nucleocapsids indicating that the various aa changed by mutagenesis were essential for some other aspect of RNA synthesis.

Experimental Design

The purpose of this project is to determine where the P protein is binding to the L protein of both measles and Sendai viruses. Site-directed mutants were created in MV 408 by Dr. Joyce Feller (University of Florida) in order to define where on the MV L protein that the P protein is binding. Mutants were constructed by site-directed mutagenesis using a charged or hydrophobic to alanine strategy in the MV 408 protein. It is thought that clustered charged aa are more likely to be on the surface of a protein and may play a role in protein-protein interactions. The hydrophobic to alanine mutants also target protein-protein interactions in that these regions may form the interface between the two proteins. The aa are changed to alanine because the small methyl side group is unlikely to disturb the tertiary structure of the protein (Cunningham and Wells, 1989; Bass et al., 1991). This strategy allowed for the identification of aa that play a

role in the MV P protein binding to the L protein. Some of the mutations were subcloned into the full length MV L protein to test for biological activity.

Based on the data obtained for MV L site-directed mutants were then constructed in the full length Sendai virus L protein. The mutagenesis strategy used to create these mutants was also clustered charge or hydrophobic to alanine but a measles virus to Sendai virus strategy was also used. Analysis of various protein-protein interactions and biological activity was used to address the consequence of the mutations.

CHAPTER 2
MATERIALS AND METHODS

Materials

Cells and Viruses

Human lung carcinoma cells (A549, American Type Culture Collection) were grown at 37^0 C with 5% CO_2 in Eagle's Minimum Essential Medium (F11, Gibco BRL) supplemented with 8% fetal bovine serum (FBS, Gibco BRL), 1% penicillin and streptomycin solution (pen/strep, penicillin 5000 I.U./ml and streptomycin 5000 µg/ml, Mediatech), 1% non-essential amino acids (100X NEAA, Mediatech), 2mM L-glutamine (200 mM, Mediatech), and 1 mM sodium pyruvate (100 mM, Mediatech). The cells were split 1:6 or 1:12 every three or four days respectively, using 1X trypsin-EDTA (ICN Biochemicals, Inc.). Baby hamster kidney cells (BHK-21, American Type Culture Collection) were maintained as A549 cells except that no sodium pyruvate was used, 0.35% glucose (17.5% glucose) was added, and FBS was at 10%. BHK-21 cells were split 1:10 or 1:20 using 1X trypsin-EDTA every three or four days, respectively.

Sendai virus (SV, Harris strain) and the Sendai virus defective interfering particle (DI-H, Harris strain) were grown in the allantoic fluid of nine day old embryonated

chicken eggs which were maintained at 37° C and were turned every 30 minutes. The eggs were then inoculated and maintained at 33° C and 85% humidity and the virus was allowed to grow for 3 days. The DI-H virus required co-infection with wild type (wt) Sendai virus which provides helper functions in trans allowing propagation of the defective interfering particle. The eggs were then placed at 4° C for 16 hours and were then incubated at -20° C for 10 minutes in order to position the allantoic fluid at the top of the egg to allow its collection. The allantoic fluid was then clarified by centrifugation at 2000 rpm on an International Equipment Company (IEC) Centra-8R centrifuge. The supernatant was then aliquoted and stored at -70° C. The viruses were purified by pelleting at 26,000 rpm for 5 hours at 4° C in a L8-55 ultracentrifuge using a SW28 rotor (Beckman) through 15 ml of 25% (v/v) glycerol in HNE buffer (10 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid)) pH 7.4, 100 mM NaCl, and 1mM EDTA (ethylenediamine tetraacetate). The pelleted virus was then resuspended in 1 ml of ET buffer (1mM EDTA and 10 mM Tris ((hydroxymethyl) aminomethane-hydrochloride (Tris-HCl)) pH 7.4) plus 10% dimethylsulfoxide (DMSO). The resuspended virus was then sonicated for 40 seconds and then further purified by banding on a 7-60% (w/w) sucrose gradient in HNE buffer at 24,000 rpm for 4° C for 17 hours in a L8-55

ultracentrifuge using a SW-41 rotor (Beckman). The virus containing bands were then diluted in ET buffer and pelleted at 30,000 rpm for 2 hours in a L8-55 ultracentrifuge using a SW-41 rotor (Beckman). The pellet was then resuspended in ET buffer plus 10% DMSO and the concentration of virus was determined by the Bradford method. The virus was further diluted to 1 μg/μl and then stored at -80^0 C in aliquots.

Stripped polymerase-free template was generated by mixing 4 ml of purified virus (1 mg/ml) with 4 ml of 2x High Salt Solubilizer(2 M NH_4Cl, 20 mM HEPES pH 8.5, 10% (v/v) glycerol, 3.7% Triton X-100, and 1.2 mM dithiothreitol (DTT)) (2x HSS) and then incubated at 4^{0}C for 2 hours. The solubilization mixture in 2 ml fractions was then layered over 2.5 ml of 30% (v/v) glycerol in 10 mM HEPES pH 8.0 and then pelleted in a L8-55 ultracentrifuge in a SW55 rotor at 50,000 rpm for 90 minutes at 4^{0}C. The pellet was resuspended in a total volume of 2 ml of 1X HSS and then incubated for 30 min at 4^{0}C. Then the mixture was split into two 1 ml aliquots which were layered over two 20-40% (w/w) CsCl gradients in ET buffer and the nucleocapsids were purified by banding in a L8-55 ultracentrifuge in a SW41 rotor at 36,000 rpm for 16 hours at 4^{0}C. The nucleocapsid band was then collected, diluted to 5 ml in 1X ET buffer and then pelleted using a L8-55 ultracentrifuge in a SW55 rotor at 50,000 rpm for 90 minutes at 4^{0}C. The pellet was

resuspended in ET buffer plus 10% DMSO to 1μg/μl. Then the nucleocapsid concentration was determined by the Bradford method.

Recombinant vaccinia virus which expresses phage T7 RNA polymerase (VVT7) was provided by Dr. Edward Niles (Suny Buffalo, NY). VVT7 was grown on ten 15 cm dishes of confluent monolayers of Vero cells at 37°C. The dishes were infected at a multiplicity of infection (MOI) of 0.05 pfu/cell. Four days post infection the cells were scraped into the medium using a rubber policeman and then pelleted at 7000 rpm for 30 minutes at 4°C in a J10 rotor (Beckman). The pellets were resuspended in a total of 10 ml phosphate buffered saline (4.3 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, and 1.5 mM potassium phosphate monobasic pH 7.2) (PBS) supplemented with 1% pen/strep. The virus was isolated from the cells by two cycles of freeze/thawing followed by sonication to disperse the membranes. The titer of the VVT7 was determined by plaque assay on A549 cells. The virus was then aliquoted and stored at -70°C.

Antibodies

The following antibodies were used for immunoprecipitation (IP) and western assays: rabbit polyclonal anti-MV L antibody (Ab) (α-MV L); and rabbit polyclonal anti-glutathione-S-transferase Ab (α-gst)

(Chandrika et al., 1995b); rabbit polyclonal anti-Sendai virus (α-SV) (Carlsen et al., 1985); rabbit polyclonal anti-SV P peptide Ab specific to the peptides from amino acids (aa) 274-298 and aa 453-477 of the P protein (α-P peptide, provided by Dr. K. Gupta, Rush Medical College, Chicago, IL). The secondary Ab used for western assays is a goat anti-rabbit immunoglobulin H+L horseradish peroxidase conjugated Ab (α-IgH+L-HRP, Southern Biotechnology Associates, Inc).

Plasmids

The following plasmids encoding the measles virus (MV) nucleocapsid (NP), phosphoprotein (P), C, and the L proteins: pBSMV-N; pBSMV-P/C; and pBSMV-L were provided by Dr. W. Bellini (CDC; Atlanta, GA) and Dr. M. Billeter (Zurich, Switzerland). pBSMV-408 is a truncation of pBSMV-L that expresses only the first 408 aa of the L protein. p107CAT plasmid encoding the chloramphenicol acetyl transferase (CAT) protein which is cloned between authentic measles virus termini was provided by Dr. S. Udem (New Jersey Medical School, Newark, NJ) (Sidhu et al., 1995). The Sendai virus plasmids: pGEMSV-NP; pGEMSV-Pstop; and pGEMSV-L which express the Sendai nucleocapsid (NP), phosphoprotein(P), and the L proteins were provided by Dr. D. Kolakofsky (Geneva, Switzerland). pGEMSV-L site1,2 is a wt pGEMSV-L plasmid with SphI and BstEII restriction enzyme

sites inserted upstream of the L protein start site was created by Dr. Joyce Feller (University of Florida). pBSSV-L-sub is a subclone of pGEMSV-L site1,2 where the SphI (blunt) and ApaI fragment containing the amino terminal 400 aa of the SV-L protein was subcloned into the SmaI (blunt) and ApaI sites of pBS. pGEMSV-Pstop contains a stop codon in the C protein open reading frame downstream of the Y2 start codon such that only the P protein is expressed. The plasmids pTM1GSTSV-P, pTM1GSTSV-C, and pTM1GSTMV-P encode amino-terminal glutathione-S-transferase (GST) fusions with the Sendai P and C proteins and the measles P protein, respectively. The pSPDI-H plasmid (Myers and Moyer, 1997) was created by RT-PCR of the DI-H genome RNA (Calain et al., 1992a) with the addition of the hepatitis delta virus ribozyme and T7 terminator at the 3' end of the DI-H DNA sequence. The ribozyme and T7 terminator sequences were provided by Dr. A. Ball (Birmingham, AL)(Pattnaik et al., 1992). All of the plasmids discussed here were cloned downstream of the T7 RNA polymerase promoter.

Construction of Mutants

Plasmid based site-directed mutagenesis

Mutagenesis of pBSMV-408 and pBSSV-L-sub was performed one of two ways, plasmid based site-directed mutagenesis or PCR based site-directed mutagenesis. The Transformer Site-Directed Mutagenesis Kit (Clontech) was used to perform the

plasmid based mutagenesis. Mutagenesis was performed on pGEMSV-L-sub because this protocol works best on small plasmids. Briefly, primers are constructed with changes in the nucleotide sequence to introduce the desired mutations as well as changes which introduce a silent restriction enzyme site. The new restriction site will be used later to select clones with the desired changes by PCR screening. In addition to the mutagenic primer another primer termed the selection primer changes a unique restriction site in the vector to another unique restriction enzyme site. In all mutants the selection primer changes a unique ScaI to StuI in the ampicillin resistance gene. In order to create a desired mutant the appropriate mutagenic primer and the selection primer were allowed to anneal simultaneously to heat denatured target vector DNA. T4 DNA polymerase was then used to elongate from the 3' end of the annealed primers which yields a double stranded plasmid with two nicks at the 5' end of each primer. T4 DNA ligase then ligates the nicks creating an intact double stranded plasmid with mismatches at the primer annealing sites. The plasmid is then digested with ScaI which will only digest wt plasmid, any plasmid with mismatches at the ScaI recognition site prevents cleavage allowing only the mutant plasmid to survive digestion. The plasmid mix is then transformed into mismatch repair deficient mut S *E. coli* (BMH 71-18 mut S

competent *E. coli*, Promega). Linearized DNA transforms bacteria at a significantly reduced level as compared to circularized intact plasmid. The intact plasmid has one strand that is wt and one strand which contains the desired mutations. The bacteria replicate the DNA in theory creating 50% mutant and 50% wt plasmid. The total plasmid DNA is isolated by mini-prep and then subjected to a second digestion with ScaI, which cleaves the wt plasmid leaving the mutant plasmid intact. The DNA was then transformed into RbCl competent UT481 *E. coli* and the transformants were then plated on LB + ampicillin (amp) agar plates and incubated overnight at 37^0 C. Individual colonies were screened by PCR using appropriate primers and the fragments were digested with the silent restriction enzyme dictated by the individual mutant. For MV 408 the plasmid was isolated from colonies with the correct digestion product and the mutants were sequenced to verify the correct sequence. For SV L the mutant in plasmid SV L-sub was isolated from a colony with the appropriate digest pattern and the insert was sequenced. Once the correct sequence was verified the mutant was digested with BstEII and ApaI and then subcloned into full length pGEMSV-L site 1,2 cut at those sites.

PCR based site-directed mutagenesis

PCR based site-directed mutagenesis utilizes four oligonucleotide primers, two outside primers (primers 1 and

2, Fig. 3) and two complementary and overlapping mutagenic primers (primers 3 and 4, Fig. 3). The mutagenic primers contain the changes required to construct the mutant as well as the changes which will insert a silent restriction site for screening. The outside primers lie up and downstream of the mutagenic primers including the restriction sites (R1 and R2) to be used to clone the PCR fragment into the appropriate vector. The first step in this mutagenesis strategy is two separate PCR reactions, the 5' outside primer (primer 1, Fig. 3) is paired with the mutagenic primer of the opposite strand (primer 3, Fig. 3) and in the other the 3' outside primer (primer 2, Fig. 3) coupled with its mutagenic primer (primer 4, Fig. 3). This leads to two PCR fragments termed the left and right arms which have complementarity where the two mutagenic primers overlap. The PCR reactions were carried out in a 25 µl reaction containing 25 pM of each primer, 50 ng of L plasmid, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% Triton X-100, 200 mM deoxynucleoside triphosphates (dNTPs) and 0.25 U Vent polymerase (New England Biolabs) in a GeneAmp PCR System 9600 machine (Perkin-Elmer) starting with 28 cycles of denaturation (94^0 C, 1 min), annealing (42^0 C, 1 min) and extension (72^0 C, 1 min). The PCR products were then separated on a 1% agarose gel (w/v) (SeaKem LE agarose) in 1x TAE (40 mM Tris-HCl pH 8.0, 20 mM acetic acid, and 1 mM

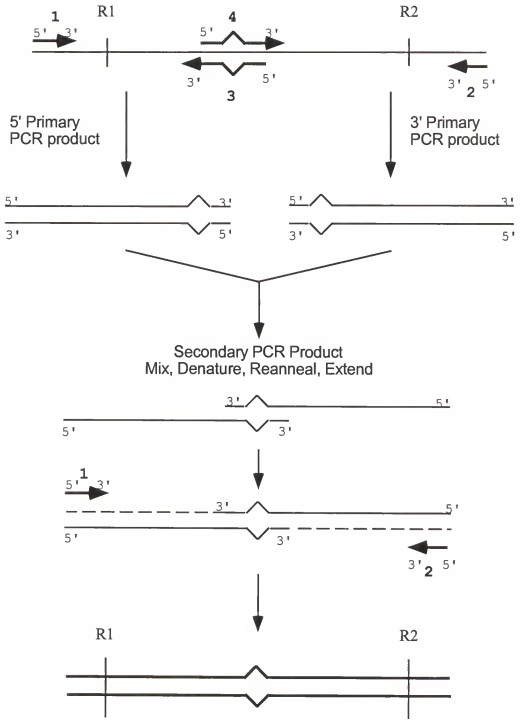


Figure 3. Methodology for PCR-based site directed mutagenesis. See Materials and Methods for a full description of the methodology.

EDTA). The DNA was stained using ethidium bromide (EtBr) (1 µl/ml) and visualized using a Ultraviolet Transilluminator (Fotodyne, Inc.) to verify that both arms were of the correct size. The arms were then removed from the gel by cutting out a small rectangle of agarose beneath the band using a scalpel. The well created by excising the agarose was filled with 15% polyethylene glycol in 1x TAE (15% PEG/TAE) and the arms were electrophoresised into the 15% PEG/TAE. The arms were then ETOH ppt using 0.1 vol of 3M NaOAc and 2.5 vol of 95% ETOH at -20^0 C overnight. The DNA was then pelleted at 13,000 RPM for 15 min at 4^0 C. The pellets were resuspended in 20 µl dH_20 and the 5' and 3' arms for each mutant were mixed in a 1:1 molar ratio and then subjected to a second round of PCR amplification. The second PCR was identical to the first reaction as described above except that the reaction was in a 50 µl reaction with the 5' and 3' arms serving as template, 50 pM of each of the outside primers (primer 1 and 2, Fig. 3) from the original PCR reaction, and 0.5 U Vent polymerase. The denatured overlapping arms are able to anneal and Vent polymerase is able to extend off the 3'-OH creating a full length, double stranded DNA fragment which serves as a template for further PCR amplification by the outside primers. The PCR product (5 µl) was then run on a 1% agarose/1x TBE (89 mM Tris-HCl pH 8.0, 2 mM EDTA, and 89 mM boric acid) gel, EtBr stained,

and visualized as before. Once the correct sized PCR product is verified the remaining 45 µl PCR product was then ETOH ppt, pelleted, and resuspended as before. The full length mutant DNA fragment was then digested with the appropriate restriction enzymes. The vector DNA was also digested with the same restriction enzymes dropping out the wt sequences. Vector DNA was the treated with shrimp alkaline phosphatase (SAP, Amersham) to prevent the vector from self-ligating without the insert sequences. The vector and insert were then ligated together using T4 DNA ligase (New England Biolabs) at 16^0 C overnight. The ligated plasmid was then transformed in RbCl competent UT481 *E. coli* and then spread onto LB + amp plates incubated overnight at 37^0 C. Colonies were then picked, touched to a replica plate, and then screened for insert using PCR. Inserts of the correct size were then digested with the silent restriction enzyme indicated for each individual mutant. A colony with an insert which gave a correct restriction pattern was grown and isolated from a mini-prep and sequenced. Once the correct sequence was confirmed the colony was grown in 500 ml of LB + amp and the plasmid was isolated using the Qiagen Mega-prep kit.

Creation of the MV 408 mutants

Dr. Joyce Feller (University of Florida, Gainesville FL) used plasmid based mutagenesis to create 12 site-

directed mutants in the pBSMV-408 plasmid. Since the MV-408 plasmid was small enough to use directly in plasmid based mutagenesis no subclone was created. The primers and the silent restriction sites used to create the individual MV 408 mutants are listed in Table 1. Table 2 lists the MV 408 mutants with the individual aa changes which are either clustered hydrophobic or charged aa to alanine or as in the case of MV 508 the substitution of a corresponding Sendai L aa for the MV aa. The mutants were all sequenced by the University of Florida ICBR sequencing core in order to verify that the correct changes were made.

Creation of the mutant full length MV L proteins

The full length MV L mutants were constructed from the MV L 408 mutants by Dr. Joyce Feller. MV 491, MV 492, MV 495, and MV 496 were selected for subcloning into full length MV L in a pBS KS+ backbone. This was accomplished using a triple ligation strategy due to the lack of unique restriction sites at the MV L start site. Three plasmids were used to generate the three fragments used in the ligation. The first plasmid is the pBS KS+ vector which was digested with SmaI (blunt) and EcoRI, generating the vector portion of the construct. The vector was then shrimp alkaline phosphatase (SAP) treated to remove the phosphates from the cleaved ends in order to prevent the vector from religating with itself. Each of the pBS MV 408 mutants was

Table 1. Oligonucleotide primers for the MV 408 alanine site directed mutants

Mutant	Mutagenic primer[a]	Enzyme
MV 491	CGATAGTTACCAATAAG**GCGGCC**GCC**GCCGCG**GAGTATGCTCGAG	NotI
MV 492	GTTGGGAATGTCATC**GCG**TCC**GCG**CTGGCCAGTTATCCGGCCC	MscI
MV 494	GGCCTAGGCTCC**GCT**CTG**GCGGCGGCC**ATCAAGGAGAAAGTTATTAAC	BsrBI
MV 495	CAGTGGTTTGAGCCG**GCTGCGGCTT**GG**GCT**ACAGTCAAGACTGAG	NgoAIV
MV 496	CACTGGTAGTTC**AGCT**GAG**GCGGCAGCC**TCTCGTGACCTTG	PvuII
MV 497	GTATATTACCTGACATTTGAA**GCCGCTGCGGCG**TATTGTGATGTCATAGAGG	BglI
MV 498	GCTAGGTATACAGAG**GCGGCC**GGCCGA**GCC**AGATACATGTGGAAAC	FseI
MV 499	GGAATCCAACTTATCAA**GCTGCA**GCC**GCGGCG**GAGCCTCTTTCAC	SacII
MV 500	GAAGCTCTAGATTAC**GCAGCTGCA**ACTGATGACATACATCTG	PvuII
MV 501	CATCTGACAGGGGAG**GCTGCC**TCA**GCTGCC**AGAAGTTTCGGCC	BbvCI
MV 502	GCAGTAACGGCTGCT**GCA**AACGTT**GCGGCA**TACATGAATCAGCC	AclI
MV 508	GAAGTTTCGGCCACCC**AAGCTT**AGAAGCAGTAACGGC	HindIII

[a]The oligonucleotide primer sequences are presented 5' to 3'. The introduced unique silent restriction site is underlined and indicated in the right column and the bold letters indicated the nucleotide changes required to create the desired mutations.

Table 2. Amino acid changes in the MV 408 mutant proteins

Mutant	Amino acid changes[a]	Type of Mutation
MV 491	I25A, V26A, I28A, L29A	H to A
MV 492	K73A, K75A, R77A	C to A
MV 494	E140A, R142A, E143A, D144A	C to A
MV 495	F175A, L176A, F177A, F179A	H to A
MV 496	V201A, L203A, L204A, I205A	H to A
MV 497	L227A, V228A, L229A, M230A	H to A
MV 498	L254A, L255A, V258A	H to A
MV 499	I279A, V280A, M282A, L283A	H to A
MV 500	I337A, F338A, I339A	H to A
MV 501	I359A, R360A, F362A, F363A	H to A
MV 502	E368A, R371A, K372A	C to A
MV 508	R360S	MV to SV

[a]Changes are indicated as the amino acid position in the MV-408 protein. The letter preceding the position is the wt sequence, and the amino acid to which it was changed appears after the number.

digested with NcoI and blunted with T4 DNA polymerase. They were then digested with BssSI generating a fragment of 617 bp which serves as one part of the insert in the construct. pBSMV-L a plasmid encoding full length MV L was digested with BssSI and EcoRI generating a fragment of 6,173 bp which will serve as the second part of the insert of the construct. The vector has a SmaI generated blunt end which is ligated with the fragment from pBS MV 408 which also has a blunted end. pBS MV 408 also has a BssSI end which is ligated to the BssSI end of the pBSMV-L fragment which also has a EcoRI sticky end which ligates with the other end of the pBS KS+ vector to create an intact plasmid. The correct sequence was confirmed by restriction enzyme digests.

Creation of the full length mutant SV L proteins

Mutagenesis to create the nine site-directed mutants in SV L used both the plasmid and PCR based mutagenesis strategies. First a subclone containing the first 5' 1200 nt of full length SV L was constructed to give more efficient mutagenesis. Full length pBS SV L site1,2 was digested with the SphI, blunted with T4 DNA polymerase and then cut with ApaI. The fragment was phenol/$CHCl_3$ extracted, ETOH ppt, and then gel purified from a 1% TAE gel. pBS KS+ serves as the vector and it was digested with EcoRV (blunt) and ApaI. The insert and vector were ligated and the plasmid used to transform RbCl competent UT481. The

plasmid was termed pBS-SV-L sub and was used in both plasmid and PCR based mutagenesis. The strategy used for the creation of these mutants was to substitute the sequence of the MV 408 mutations into the corresponding positions of the SV L protein. The mutants with the correct sequence were subcloned back into full length SV L site 1,2 using the restriction enzymes BstEII and ApaI. Table 3 lists the SV L mutants created by the plasmid based method including the mutagenic primer and the engineered silent restriction site used to select for mutants during PCR screening. Mutant 519 and 521 were created using the PCR based strategy using the T7 and T3 promoter sequences as the outside primers and the PCR fragment was subcloned into SV-L using the restriction enzymes BstEII and NcoI. Table 4 lists the sense and antisense mutagenic primers and the engineered silent restriction site used to create the mutants SV 519 and SV 521. Table 5 summarizes the amino acid changes of all the SV-L mutants.

Methods

Protein Analysis

Infection and transfection

A549 cells were incubated overnight at 37^0 C in 35 mm or 60 mm dishes to an approximate subconfluent density of 1.6×10^6 or 5×10^6 cells per dish, respectively. Cells were then infected with VVT7 at an m.o.i. of 2.5 pfu/cell at

Table 3. Oligonucleotide primers for plasmid based SV L site directed mutagenesis

Mutant	Mutagenic primer[a]	Enzyme
SV 514	CTATCCAGAATGCCAC**GC**GAAC**G**CTCC**GGCCG**C**C**AGGGGGAAGATAGC	EagI
SV 515	CATAGTCAGGGGGAAG**GC**AGCA**GC**G**GCGGCC**G**TCTTGTTAGATGTG	EagI
SV 516	GTCTCTGGGTAAGGC**GCTAGC**ATCC**G**CAATAGCGGATTTAGACCGATAC	NheI
SV 517	GAATAGATGGTATAGGCCA**GCTGCAG**CTTGG**GC**CAGCATCAAATATGAC	PstI
SV 518	CCTAGAATGCAAATC**AGCTGAGGC**AG**CAG**CATACGGAGATCTTG	PvuII
SV 520	GATAAGAAGTCCATTGGG**GCGGCCG**GCA**G**AG**C**TGAGGAATTATGGGAAC	EagI
SV 522	GAGTCGTTACTCGCC**GCGGCCGC**TGGAACCTCTATTGATG	EagI

[a]The oligonucleotide primer sequence are presented 5' to 3'. The introduced unique silent restriction site is underlined and indicated in the right column and the bold letters indicated the nucleotide changes required to create the desired mutations..

Table 4. Oligonucleotide primers for PCR based SV L site directed mutagenesis

Mutant	Mutagenic primers[a]		Enzyme
SV 519	SM 519 (+)	GTATATCCTAACCCCTGAG**GC**GGCCG**C**G**GC**GTATTGTGATGTTGTAG	EagI
	SM 527 (-)	CATCACAATACGCCGCGGCCGCCTCAGGGGTTAGGATATACC	
SV 521	SM 521 (+)	GAGAGGAAATATACAATG**C**GGC**C**G**C**A**GC**A**GC**GGAGCCCCTATCAC	EagI
	SM 529 (-)	GTGATAGGGGCTCCGCTGCTGCGGCCGCATTGTATATTTCCTCTCCAAG	

[a]The oligonucleotide primer sequence are presented 5' to 3'. The introduced unique silent restriction site is underlined and indicated in the right column and the bold letters indicated the nucleotide changes required to create the desired mutations.

Table 5. Amino acid changes in SV L site1,2 mutant proteins

Mutant	Amino acid changes[a]	Type of mutation
SV 514	L20A, S22A, I24A, V25A	H to A
SV 515	T29A, Q31A, L32A, H33A	C to A
SV 516	Q77A, R78S, T79A, K81A	C to A
SV 517	F173A, L174A, T175A, F177A	H to A
SV 518	Y209A, L210E, T211A, V212A, T213A	C to A
SV 519	L235A, V236A, L237A, M238A	H to A
SV 520	L262A, L263A, V264G, K265R, G266A	H to A
SV 521	V287A, I288A, L290A, L291A	H to A
SV 522	I345A, F346A, H347A	H to A

[a]Changes are indicated as the amino acid position in the SV-L site1,2 protein. The letter preceding the position is the wt sequence, and the amino acid to which it was changed appears after the number.

37^0 C for 1 hour in an inoculum of 0.3 ml for a 35 mm dish and 0.9 ml for a 60 mm dish of F11 adsorption media (F11 medium supplemented with 1% pen-strep, 2 mM L-glutamine, and 14 mM HEPES pH 7.4). The dishes were rocked at 30 minutes and the inoculum was removed at 1 hour post-infection. The cells were then washed once with 1 ml of unsupplemented Opti-MEM. Opti-MEM supplemented with 1% pen-strep was then added to the dish, 0.8 ml for 35 mm and 2.5 ml for a 60 mm dish. The cells were transfected using a ratio of 3 µl of Lipofectin to 1 µg of plasmid DNA in supplemented Opti-MEM in a total volume of 0.2 ml. This transfection mix was incubated at room temperature (RT) for 30 minutes prior to addition to the washed and infected cells.

In vitro protein synthesis

All of the mutants were tested for full length protein expression in an *in vitro* coupled transcription and translation system (TNT Coupled Reticulocyte Lysate System, Promega). As instructed in the manufacturers protocol 1 µg of plasmid was added to the TNT reaction mix containing rabbit reticulocyte lysate, T7 RNA polymerase buffer, T7 RNA polymerase and 4 µCi of L-[4,5-^{3}H]leucine (1 mCi/ml, 157 Ci/mmol, Amersham) in a total volume of 12 µl. The reaction mix was then incubated for 2 hours at 30^0 C. Half of the sample was mixed with 6 µl of 2x LB (4% sodium dodecyl

sulfate (SDS), 50 mM Tris-HCl pH 6.8, 0.2 M DTT, 40% glycerol, and 0.1% bromophenol blue (BPB)), boiled for 2 minutes and then separated on a 7.5% SDS-polyacrylamide gel (SDS-PAGE). For fluorography the gel was washed once in 1x used DMSO for 30 minutes, once in new DMSO for 30 minutes, incubated in 22% (w/v) 2,5-diphenyl-oxazole in DMSO (PPO/DMSO) for 30 minutes, and then rehydrated in H_2O for 30 minutes with frequent changes with fresh H_2O. The gel was then dried for 1 hour at 80^0 C using a slab gel dryer (SE1160, Hoeffer Scientific Instruments). The dried gel was then exposed to Kodak X-Omat film to visualize the labeled proteins by autoradiography.

In vivo protein synthesis

Protein expression and stability *in vivo* was determined by metabolic labeling in infected/transfected A549 cells with Express-[^{35}S] protein labeling mix (10 mCi/ml ^{35}S-methionine, 1175 Ci/mmol, Dupont NEN). The transfection went for Five hr in an overnight experiment or 10 hr in a short term experiment. The cells were labeled overnight or for 30 min with 150 µCi/ml of Express-[^{35}S] in label media (cysteine and methionine free medium (Mediatech) plus 1% pen-strep, 2 mM L-glutamine, and 14 mM HEPES pH 7.4) plus 0.1 volume of F11 to provided unlabeled cysteine and methionine at 0.1x, at 0.75 ml in a 35 mm dish or 1.5 ml in a 60 mm dish. The cells were then washed once with cold

PBS, dishes were placed on their edge to drain and the remaining wash was aspirated. Cell extracts were prepared using 1% Nonidet P-40 (NP-40) lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, and 1 µg aprotinin). Cells were scraped into 100 µl of NP-40 lysis buffer using a rubber policeman. The cell suspension was transferred to a microfuge tube, vortexed for 30 seconds, and cell debris was pelleted at 13,000 RPM for 30 minutes at 4^0 C. The supernatant was removed to a fresh microfuge tube and 5 µl of cell extract was mixed with 5 µl of 2x LB, boiled for 2 minutes, and then separated by 7.5% SDS-PAGE. The labeled proteins were visualized by fluorography and autoradiography.

Immunoprecipitation assay

Immunoprecipitation (IP) assays are performed on cells infected and transfected as for *in vivo* protein expression except the radiolabeled cell extracts are scraped into 150 µl of SV salts (0.1 M HEPES pH 8.5, 0.05 M NH_4Cl, 7 mM KCl, 4.5 mM MgOAc) with 0.25% NP-40, and 1 µg/ml aprotinin. Cell debris was pelleted at 13,000 RPM for 30 min at 4^0 C. The cleared lysate was then divided into 100 µl and a 50 µl samples. From the 50 µl sample, 10 µl was analyzed by a 7.5% SDS-PAGE gel to show total protein expression. For the IP, the 100 µl sample of cleared lysate was brought up to

250 µl by the addition of SV salts + 0.25% NP-40, then 50 µl of *Staphlococcus aureus* (*S. Aureus*, Cowen strain, ATCC) washed in SV salts + 1% NP-40 was added to the cell lysate. Preabsorption of the extract was then performed by incubating the extract for 30 min at 4^0 C. The *S. aureus* was then pelleted in a Costar microfuge for 3 min and the supernatant was transferred to a new microfuge tube. The sample was incubated with the appropriate antibodies for 1 hr at 4^0 C to allow formation of Ab-antigen complexes. *S. aureus* (50 µl) was then added to the cell extract and the antibody-antigen complex binds to protein A expressed on the bacteria via the Fc portion of the Ab. The complexes are then pelleted using a Costar microfuge and were then washed twice with 1 ml SV salts + 1% NP-40. The pellet was resuspended in 40 µl 2x LB, boiled 2 min, and then analyzed by 7.5% SDS-PAGE. The protein was visualized by fluorography and autoradiography.

Glutathione-Sepharose bead binding assay

The fusion proteins gstP for both measles and Sendai viruses and gstC for Sendai virus were used in a glutathione Sepharose bead binding assay to measure complex formation with wt or mutant measles or Sendai L proteins. From a cleared radiolabeled cell lysate, prepared as above, 10 µl was analyzed by 7.5% SDS-PAGE to show total protein

expression. A 100 µl aliquot of clarified cell extract was diluted to 450 µl in SV salts + 0.25% NP-40. Gutathione-Sepharose beads were blocked for 15 min in SV salts plus 0.1% NP-40, 0.5% nonfat dairy milk (NFDM), and 10 mg/ml bovine serum albumin (BSA). The beads were pelleted in a Costar microfuge for 2 min, washed twice in SV salts, and then resuspended in 50 µl per reaction in SV salts + 0.25% NP-40. Beads were incubated with the cell extracts for 15 min at 4^0 C with vortexing every 5 min. The beads were pelleted in a Costar microfuge for 1 min, the supernatant was aspirated and the beads were then washed 3x in 1 ml SV salts + 0.25% NP-40 per wash. After the final wash the beads were pelleted and then resuspended in 20 µl 2x LB, boiled 2 min, and the proteins were separated by 7.5% SDS-PAGE and visualized by fluorography and autoradiography.

Western blot assay

The unlabeled cell extracts used in this assay are described under the RNA synthesis section. The cell extract (4-10 µl) was mixed 1:1 with 2x LB and then separated by 7.5% SDS-PAGE. The gel, 6 pieces of 3 mm paper (Whatman), and nitrocellulose membrane (Protran, Schleicher and Schuell) were equilibrated in 1x transfer buffer [190 mM glycine, 25 mM Tris, and 20% (v/v) methanol]. The separated proteins were then blotted onto the nitrocellulose membrane

at 40V for 18 hrs at 4^0 C in 1x transfer buffer using the Mini-Protean electrophoresis apparatus (Biorad). The nitrocellulose membrane was washed once in TBS + Tween 20 (0.02 M Tris pH 7.5, 0.5 M NaCl, and 0.05% (v/v) Tween 20) and incubated with rocking in 40 ml blocking buffer (5% NFDM in TBS + Tween 20) for 1 hr at RT. The blot was washed twice in TBS + Tween 20 for 5 min and once for 15 min. The blot was then incubated for 1 hr at RT with the appropriate Ab which was diluted 1:5,000 or 1:10,000 in 20 ml TBS + Tween 20 supplemented with 2.0% BSA and 0.01% sodium azide. The Ab dilution was removed and saved for reuse and the blot was washed twice in TBS + Tween 20 for 5 min and once for 15 min. The blot was then incubated for 2 hr at 4^0 C with 10 ml of blocking buffer supplemented with 4 µl secondary Ab (goat anti-rabbit Ig(G+L)-HRP), washed twice in TBS + Tween 20 for 5 min and once for 15 min and the proteins were visualized using Enhanced Chemiluminescence (ECL) Plus protein identification system (Amersham). Following mixing 975 µl of solution 1 with 25 µl solution 2 and addition to the washed blot the horseradish peroxidase catalyzes a reaction which generates light which is detected by autoradiography.

Nucleocapsid binding assay

The nucleocapsid binding assay uses co-sedimentation to measure the ability of wt and mutant polymerase complexes to

bind viral nucleocapsids. A549 cells in 100 mm dishes were infected with VVT7 and transfected with 15 μg of Sendai P plasmid and 15 μg of wt or mutant Sendai L plasmid. Five hours post-transfection the cells were labeled with Express-[^{35}S] protein labeling mix and then incubated overnight at 37^{0} C. The cell were washed once with 6 ml of wash solution (150 mM sucrose, 30 mM HEPES pH 7.4, 33 mM, NH_4Cl, 7 mM KCl, and 4.5 mM MgOAc) per dish at 4^{0} C. The wash solution was then aspirated and cell extracts were prepared using lysolecithin (L-α-lysophosphatidylcholine palmitoyl, Sigma) permeabilization at 4^{0} C. Lysolecithin (2.5 ml of 100 μg/ml) was added to the dishes and they were rocked continuously for 1 min. Wash solution (6 ml) was added to dilute the lysolecithin, aspirated and the dishes were placed on their edge for 4 min to allow them to drain and were then aspirated. The cells were scraped in 320 μl of SV reaction mix salts (0.1 M HEPES pH 8.5, 50 mM NH_4Cl, 7 mM KCl, 1mM DTT) + 1mM ATP. The cell suspension was transferred to a microfuge tube and pipetted up and down 15 times to break open the cells. The cytoplasmic cell extracts were clarified by centrifugation at 13,000 RPM for 30 min at 4^{0} C. Two 125 μl aliquots of the cytoplasmic extract were incubated with and without polymerase free wt nucleocapsids (NC) (1 μg) for 1 hr at 30^{0} C. From the

remaining extract 10 µl was separated by 7.5% SDS-PAGE to determine total protein expression. The extracts were then pelleted through a step gradient of 2 ml 30% and 2.5 ml 50% glycerol (w/v) in 10 mM HEPES pH 8.5 plus 1 mM EDTA at 45,000 RPM for 75 min at 4^0 C in a SW55 rotor. The pellets were resuspended in 20 µl 2x LB, separated by SDS-PAGE and the proteins visualized by fluorography and autoradiography.

Chloramphenicol acetyl transferase (CAT) assay

In measles virus using a CAT minigenome reporter system the wt and mutant polymerase complexes were assayed for their ability to perform transcription and replication in order to yield CAT activity. BHK cells (60 mm dishes) were VVT7 infected as described before and transfected in triplicate with 2.5 µg p107CAT, 2.5 µg pBSMV-NP, 5 µg pBSMV-P, and wt or mutant 0.5 µg pBSMV-L. The cells were washed 24 hr post-transfection in 2 ml PBS and scraped into PBS using a rubber policeman. The cells were pelleted at 1600 RPM for 5 min at 4^0 C and then resuspended by vortexing in 50 µl of 0.25 M Tris-HCl pH 7.8 + 0.5% Triton X-100. The extracts were incubated at 4^0 C for 10 min and then at 65^0 C for 10 min to remove any background activity intrinsic to the BHK cells. The cell extracts were pelleted at 13,000 RPM for 30 min at 4^0 C and the supernatant assayed for CAT activity. A 7 µl aliquot was removed for western analysis

and 40 µl was removed for analysis of CAT activity. For each sample 20 µl of 8 mM chloramphenicol (1.6 mM final), 20 µl of 0.25 M Tris-HCl pH 7.8, and 20 µl of ^{14}C acetyl CoA (10 mCi/ml diluted 1:1 to 5 µCi/ml in 0.25 M Tris-HCl pH 7.8) was added and incubated at 37^0 C for 3 hr. The extracts were incubated at 4^0 C and extracted with 100 µl cold ethyl acetate by vortexing for 1 min and the layers separated using a Costar microfuge. The top organic layer (80 µl) and 100 µl of a second extraction were added together and counted in a LS8000 scintillation counter (Beckman).

RNA Synthesis

Transcription assay

Subconfluent A549 cells in 60 mm dishes were infected with VVT7 and then the cells were transfected overnight with pGEMSV-Pstop (1.5 µg) and wt or mutant pGEMSV-L (0.5 µg). Cell extracts were prepared by lysolecithin permeabilization as described above except that the cells were washed with 3 ml of wash solution and permeabilized with 1 ml lysolecithin. Cell extracts were prepared by scraping into 110 µl of SV incomplete reaction mix (RM) (100 mM HEPES pH 8.5, 50 mM NH_4Cl, 7 mM KCl, 4.5 mM MgOAc, 1 mM DTT, 1 mM spermidine, 1mM ATP, 1mM GTP, 1mM UTP, 10 µM CTP, and 10%

glycerol). The cell suspension was then transferred to a microfuge tube and pipetted up and down 15 times to break open the cells. Nuclei and other cellular debris were pelleted by centrifugation at 1600 RPM for 5 min at 4^0 C. Western analysis was performed on 10 µl of the supernatant and the remaining extract was incubated with 1 mM $CaCl_2$ and 20 µg/ml micrococcal nuclease (MN) for 30 min at 30^0 C to remove DNA and RNA from the extract. The MN was inactivated by chelation of Ca^{++} by the addition of 2.2 mM EGTA (ethylene glycol-bis(aminoethyl ether)-N,N-tetraacetic acid pH 8.0). The nuclease treated extracts were supplemented with 0.1 vol 10x supplemental mix (45 mM MgOAc, 5 U/µl RNasin, 200 µg/ml actinomycin D, 400 U/ml creatine phosphokinase (CPK), and 33 mg/ml creatine phosphate), 1 µg polymerase-free wt Sendai virus template, and 20 µCi of [α-^{32}P]CTP (10 mCi/ml, 3000 Ci/mmol, Dupont NEN). The samples were incubated at 30^0 C for 2 hr and the RNA was isolated using the Qiagen Rneasy Total RNA kit as instructed in the manufacturer's protocol. The RNA was precipitated with an equal vol of 5 M NH_4OAc and 2.5 vol 95% ETOH. The RNA was frozen on dry ice for 15 min, thawed, and pelleted at 13,000 RPM for 30 min at 4^0 C. The supernatant was decanted and the interior of the microfuge tube was wiped dry with a sterile cotton swab and then allowed to air dry for 10 min

at RT. The RNA pellet was resuspended in 10 µl loading buffer (2.5 mM citrate buffer pH 3.5, 6 M urea, 20% sucrose, 5 mM EDTA, and 0.012% BPB), boiled for 2 min, quenched on ice, and separated on a 1.5% agarose/6 M urea-citrate gel. The gel was washed overnight in 7% acetic acid to remove the urea and then dried onto 3 mm filter paper for 1 hr at 40^0 C and then 1 hr at 80^0 C. The mRNA was visualized by autoradiography and quantitated using a phosphorimager (Storm 850, Molecular Dynamics).

Leader RNA assay

Leader RNA is the first product of transcription and is measured in the *in vitro* assay by Northern blot analysis. An extract of infected, transfected cells was prepared as for the transcription assay described above except that the cells are scraped into SV incomplete RM with 1 mM CTP and RNA synthesis occurs in the absence of radiolabeled nucleotides. After the 2 hr incubation at 30^0 C the samples are treated with NENSH (0.1 M NaCl, 0.05 M NaOAc, 0.01 M EDTA, 0.5% SDS and proteinase K at pH 5.1) at 30^0 C for 30 min. The samples were then extracted twice with phenol/$CHCl_3$, and ETOH precipitated. The pelleted RNA was resuspended in 15 µl stop mix (94% formamide, 12.5 mM EDTA, 0.3 mg/ml xylene cyanol, and 0.3 mg/ml BPB), boiled for 2 min, quenched on ice, and then separated on a prerun (400 V for 1 hr) 8% polyacrylamide/8 M urea vertical gel at 400 V

until the BPB dye is 2/3 of the way into the gel. The gel was then washed 4 times in 250 ml 1x TBE with shaking for 15 min each wash. The RNA was electroblotted onto Hybond-N-nitrocellulose (Amersham) in a semi-dry blotter (Fisher Scientific) for 1 hr at contant milliamperes (mA) as indicated by the size of the blot (blot size in cm^2 x 0.8 mA). The membrane was then washed for 5 min in 5x SSC ((1x SSC) 0.75 M NaCl and 75 mM sodium citrate). The membrane was removed to a piece of 3mm paper wet with 5x SSC and the RNA was covalently crosslinked (Stratalink UV Crosslinker 1800, Stratagene) to the membrane. Prehybridization buffer (50% formamide, 4x Denhardts (1% (w/v) Ficoll, 1% (w/v) polyvinyl pyrolidone, and 1% (w/v) bovine serum albumin), 0.05% SDS, 200 µg/ml salmon sperm DNA, 200 µg/ml tRNA, and 2.5x SSPE (0.9 m NaCl, 2.5 M NaOH, 5 mM EDTA, 50 mM sodium phosphate pH 7.4)) was boiled for 5 min and then quenched on ice. The blot was then incubated with the prehybridization buffer at 40^0 C for 2 hr in a shaking water bath incubator (Gyrotory Water Bath Shaker, New Brunswick Scientific). Then a ^{32}P end labeled oligo (described below) was added to the prehybridization buffer and allowed to incubate with the membrane for 18 hr at 40^0 C. The blot was then rinsed briefly once in 6x SSPE/0.1% SDS, washed three times in 6x SSPE/0.1% SDS for 15 min at RT, washed once in 6x SSPE/0.1% SDS for 15 min at 40^0 C, and finally once in 6x SSPE without

SDS for 15 min at 40° C. The blot was then wrapped in plastic wrap, visualized by autoradiography, and quantitated with a phosphorimager.

The probe used in this experiment to detect leader RNA was the oligonucleotide designated SM04 with the sequence (5'AAATCCTGTA TAACTTCATT ACATATCCCA TACATGTTTT TTCTCTTGTT TGGT3'). This oligo is genomic (-) sense so it is complementary to the leader RNA product (le^+). The probe was end-labeled with γ-^{32}P ATP (10 mCi/ml, 3000 mCi/mmol, Dupont NEN) using T4 polynucleotide kinase (New England Biolabs).

In vitro replication assay

A549 cells (60 mm dish) were infected as before and transfected with pGEMSV-Pstop (5 µg), pGEMSV-NP (2 µg), and the wt or mutant pGEMSV-L (0.5 µg). Cell extracts were prepared after 20 hr as in *in vitro* transcription except that the cells were scraped in SV incomplete RM supplemented with 4.5 mM MgOAc and 0.5 U/µl RNasin. Cell debris was pelleted by centifugation at 1600 RPM for 5 min at 4° C. A 10 µl aliquot was removed for western analysis and the remaining extract was supplemented with 20 µg/ml actinomycin D, 50 µCi [α-^{32}P]CTP, and 2.5 µg polymerase-free DI-H template. The extract was incubated at 30° C for 2 hr to allow replication to occur. The samples were treated with

MN as before and the MN was inactivated by the addition of 15 mM EGTA. RNA was isolated using Qiagen Rneasy Total RNA kit, precipitated, and resuspended in 10 µl loading buffer and then separated on a 1.5% agarose/6 M urea gel. The gel was dried as described before and the RNA was visualized by autoradiography and quantitated on a phosphorimager.

In vivo replication assay

A549 cells were infected with VVT7 and transfected with pGEMSV-NP (2 µg), pGEMSV-Pstop (5 µg), wt or mutant pGEMSV-L (0.5 µg), and pSPDI-H (2.5 µg). Cell extracts were prepared as discussed before using lysolecithin permeabilization. The extracts were treated with MN and the nuclease resistant product RNA which was encapsidated by NP was directly isolated using Qiagen RNeasy Total RNA Kit. The RNA was separated by elecrophoresis on a 1.5% agarose/6 M urea gel and the unlabeled DI-H replication product was detected by Northern blot analysis with a DI-H specific probe. The probe was created using pSPDI-H plasmid which was linearized with the restriction enzyme XbaI. The linearized plasmid was transcribed with the T7 RNA polymerase and was internally labeled by the addition of [α-^{32}P]CTP to the transcription reaction. The positive sense RNA probe was used to detect the negative sense DI-H replication product. After the washes the blot was wrapped in plastic wrap and

the RNA was visualized by autoradiography and quantitated on the phosphorimager.

CHAPTER 3

MEASLES AND SENDAI VIRUS L PROTEINS: INTERACTIONS WITH THE HOMOLOGOUS VIRAL P PROTEIN AND FORMATION OF AN ACTIVE POLYMERASE COMPLEX

Introduction

The current model for RNA synthesis is that polymerase complex formation in negative strand RNA viruses is absolutely critical for the virus life cycle. It is thought that the P and L proteins together form the polymerase complex and that only this complex is capable of carrying out viral replication and transcription. The model suggests that the P protein of the polymerase complex actually contacts the nucleocapsid template and that the L protein encodes all catalytic functions required for both replication and transcription. It is known that in Sendai virus the binding of the P protein stabilizes the expression of the L protein on the order of 10 fold. Studies with truncations of the L proteins of measles and Sendai viruses show that carboxy-terminal truncations can still bind to the P protein. In measles virus only the amino-terminal 408 aa of the L protein are required for P protein binding. In Sendai virus the amino-terminal 1147 aa seem to be required as any shorter truncation loses the ability to bind to the P

protein (Chandrika et al., 1995a). Our hypothesis for both measles and Sendai viruses is that the P binding site on the L protein resides within the first 400 aa of the L protein and thus this portion of L is required for polymerase complex formation. Further we hypothesize that without binding of the P protein, the L protein will be unstable and it will be unable to utilize the viral template. Any L protein unable to bind the P protein will, therefore, be deficient in viral replication and transcription. This work will show that the P binding site resides within the amino terminal 400 aa of both measles and Sendai viruses. It will also show that P binding to the L protein is required for viral transcription and replication. The mutations in the amino terminus of the L proteins show a spectrum of binding defects to the P proteins and in addition those L proteins that partially bind to the P protein have partial activity in transcription and replication.

Results

Measles Virus

MV 408 mutant protein expression in the TNT system

MV 408 is a carboxy-terminal truncation of full length MV L. This truncated L protein expresses the first 408 aa of the wt MV L protein (Chandrika et al., 1995b). To identify amino acids important for binding to the MV P protein, twelve site-directed mutants throughout MV 408 were

constructed by Dr. Joyce Feller (University of Florida) using an alanine scanning mutagenesis strategy (Tables 1 and 2). The mutant plasmid DNAs were then tested for their ability to synthesize full length protein in an in vitro coupled transcription and translation (TNT) assay. The proteins were analyzed by SDS-PAGE as detailed in Materials and Methods (Fig. 4). No protein synthesis was detected in the absence of added plasmid (Fig. 4, lane 1). Compared to the synthesis of wt MV 408 (Fig. 4, lane 2) each of the twelve mutants was synthesized at similar levels and all mutants gave a full length protein product (Fig. 4, lanes 3-14). It is apparent that many of the mutants, including MV 494, MV 495, and MV 499 (Fig. 4, lanes 5, 6, and 10) gave proteins with slower mobilities. Each gene was sequenced to confirm that each mutant encodes for a protein of 408 aa. Since these mutants were created using an alanine scanning mutagenesis strategy, the altered mobilities of the proteins are most likely due to changes in SDS binding. It is this change in SDS binding which can lead to changes in protein mobility on a SDS-PAGE gel.

MV 408 mutant protein binding to the MV gstP protein

Once it was shown that the MV 408 mutants all synthesized full length protein, their ability to bind to the MV gstP protein and to form a polymerase complex was tested. The mutant and wt MV 408 proteins were co-expressed

Figure 4. Coupled transcription and translation of wt or mutant MV 408 plasmids using the TNT Coupled Reticulocyte Lysate System. T7 RNA polymerase, [^{3}H]-lysine, and 0.5 µg of wt or mutant MV 408 plasmid were incubated at 30^0 C for 2 hrs with a rabbit reticulocylte lysate. The resultant protein product was visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein is indicated on the left of the gel.

MV 408-
Mock
MV 408
MV 491
MV 492
MV 494
MV 495
MV 496
MV 497
MV 498
MV 499
MV 500
MV 501
MV 502
MV 508
1 2 3 4 5 6 7 8 9 10 11 12 13 14

with the MV gstP protein and were tested for binding to glutathione conjugated Sepharose beads in a binding assay. Mammalian cells were infected with VVT7 to provide cytoplasmic expression of T7 RNA polymerase. The cells were then transfected with the MV gstP plasmid together with the wt or mutant MV L plasmids where each gene is under the control of the T7 promoter. The cells were radiolabeled with ^{35}S Express label overnight and cytoplasmic extracts were prepared as discussed in Materials and Methods. The extracts were incubated with glutathione beads and the bound proteins were analyzed by SDS-PAGE. The mock transfected sample showed the absence of viral or host proteins bound to the beads (Fig. 5, lane 1). MV gstP protein binding was fairly uniform in each sample (Fig. 5, lanes 2-14). The wt MV 408 protein bound well to the MV gstP protein showing that the wt truncated MV L protein retained its ability to form a complex with the gstP protein (Fig. 5, lane 2). The wt MV 408 protein from the TNT (Fig. 4) described above was run as a size marker (Fig 5, lane 15). The mutant MV 491 protein bound to the MV gstP protein only at the limits of detection (Fig. 5, lane 3). Mutants MV 494, MV 501, MV 502, and MV 508 were all able to bind to the MV gstP protein and form a complex at significant levels (Fig. 5, lanes 5 and 12-14). More importantly the mutants MV492, MV 495, MV 496, MV 497, MV 498, MV 499, and MV 500 all failed to bind to the

Figure 5. Bead binding assay to measure complex formation between MV gstP and wt or mutant MV 408 proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 1 μg of MV gstP and 1.67 μg of wt or mutant MV 408 as indicated above each lane. The cells were incubated with ^{35}S-EXPRESS for 18 hrs and then cytoplasmic extracts were prepared as detailed in the materials and methods section. The extracts were then incubated with glutathione conjugated Sepharose beads and then washed. The bound proteins were visualized by fluorography on a 7.5% SDS-PAGE gel. Lane 15 is protein product from the TNT (Fig. 4) run as a size marker. The position of the protein products are indicated on the left of the gel.

gst P-
MV 408-
Mock
MV 408
MV 491
MV 492
MV 494
MV 495
MV 496
MV 497
MV 498
MV 499
MV 500
MV 501
MV 502
MV 508
MV 408
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

MV gstP protein (Fig. 5, lanes 3, 4, and 6-11), showing that all of these amino acids are important for the interaction with the MV P protein. The MV 501 mutant protein appears to give different mobilities in the TNT and bead binding assays, this is most likely due to the abnormal distortion (smiling) present in the bead binding gel (Fig. 4, lane 12 and Fig. 5, lane 12). Normally this experiment includes an IP which allows us to monitor how the various total proteins were expressed, in the event that one of the mutant proteins failed to bind to MV gstP protein in the bead binding assay. Since we do not have an antibody that will immunoprecipitate the MV 408 protein or the various mutants, protein synthesis was shown to occur instead in the TNT assay discussed above (Fig. 4).

Subcloning of selected MV 408 mutants into full length MV L protein

The next step was to subclone into the full length MV L plasmid some of the MV 408 mutants which failed to form a complex with the MV gstP protein. This will allow us to determine if the binding effect is also true in the full length MV L mutant proteins. In order to have the full length MV L mutants in a vector backbone compatible with the MV gstP vector a triple ligation procedure was used as described in Materials and Methods. Only a few of the MV 408 mutants had restriction enzymes sites suitable for

subcloning. MV 491, MV 492, MV 495, and MV 496 were ligated into full length MV L by Dr. Joyce Feller (University of Florida) and I carried out the transformations, identification, and characterization of the clones. The full length MV L clones are designated FL 491, FL 492, FL 495, and FL 496, respectively. Expression of these plasmids in the TNT system showed that the full length proteins were all synthesized.(data not shown).

Complex formation of the full length MV L mutants with MV gstP protein

The wt and mutant full length MV L proteins were tested for expression and complex formation with the MV gstP protein in mammalian cells. The cells were infected with VVT7 to provide cytoplasmic expression of the T7 RNA polymerase. The cells were then transfected with the MV gstP plasmid together with the wt or mutant full length MV L plasmid where each gene is under the control of the T7 promoter. The cells were incubated with ^{35}S Express label overnight, cytoplasmic extracts were prepared and then divided into two aliquots as described in Materials and Methods. One aliquot was incubated with the α-MV L and α-gst antibodies. The immunocomplexes were then pulled down by the addition of *S. aureus*. The bound proteins were analyzed by SDS-PAGE (Fig. 6A). The mock transfected sample showed some vaccinia virus and/or host background proteins

which are nonspecifically immunoprecipitated (Fig. 6A, lane 1). When the wt MV L plasmid was transfected alone, only small amounts of the full length protein were detected (Fig. 6A, lane 2), however, when MV gstP and wt MV L plasmids are transfected together expression of the L protein was increased (Fig. 6a, lane 3). The MV gstP protein migrated at the same position as one of the vaccinia virus or host background proteins and
was distinguished only as a slight increase in the intensity of that band (Fig. 6A, lanes 3-7). Expression of the full length MV L mutants with the MV gstP protein showed only a small amount of each of the L mutant full length proteins (Fig. 6A, lanes 4-7). The levels of these mutants more closely resembled that of wt MV L protein alone and is suggestive that these L mutants are not binding to the MV gstP protein. It is known that in other Paramyxoviruses that L-P polymerase complex formation is required for the stability of the L protein. These data suggest that it is also true in measles virus that the L protein requires coexpression of the P protein for stability.

The bead binding assay was performed on the second half of the cell extract and analyzed by SDS-PAGE (Fig. 6B). The mock transfected sample showed that there were no vaccinia virus or host background proteins capable of binding to the glutathione beads, nor did the wt MV L protein when it was

Figure 6. Immunoprecipitation and bead binding assay to measure complex formation between MV gstP and full length, wt or mutant MV L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 1 μg of MV gstP and 1.67 μg of wt or mutant MV L plasmids as indicated above each lane. The cells were incubated with ^{35}S-EXPRESS for 18 hrs and then cytoplasmic extracts were prepared as detailed in the materials and methods. The extract was then divided into two equal aliquots and (**A**) immunoprecipitated with α-MV-L and α-gst antibodies or (**B**) incubated with glutathione conjugated Sepharose beads and then washed. The bound proteins were visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein bands are indicated on the left of the gel.

A
Mock
MV-L
L + gstP
FL 491
FL 492
FL 495
FL 496
L -
gst P -
1 2 3 4 5 6 7
B
Mock
MV-L
L + gstP
FL 491
FL 492
FL 495
FL 496
L -
gst P -
1 2 3 4 5 6 7

expressed alone (Fig. 6B, lanes 1 and 2). When wt MV L and MV gstP plasmids were transfected together a complex was formed which was capable of binding to and coming down with the glutathione beads (Fig. 6B, lane 3). In this experiment the presence of MV gstP was unambiguous due to the absence of the vaccinia virus background proteins and it was shown that MV gstP is bound well in each smaple (Fig. 6B, lanes 3-7). The mutant FL 491 protein when co-expressed with the MV gstP protein gave weak binding as compared with the wt MV L protein (Fig. 6B, lane 4). It is of note that in the bead binding experiment with the cognate truncated MV 408 mutant, MV 491 gave a similar hint of binding (Fig. 5, lane 4). Fl 492, Fl 495, and Fl 496 when expressed with the MV gstP protein gave no detectable complex formation which is analogous to the lack of binding of their MV 408 correlates (Fig. 6B, lanes 5-7). Therefore, unlike the wt MV L protein, none of these mutant full length MV L proteins were able to form a polymerase complex.

CAT assay to test the mutant full length measles virus L mutants for their ability to perform *in vitro* RNA synthesis

The functional activity of the mutant and wt full length MV L proteins were then tested. The measles virus CAT assay is a mini-genome rescue system that tests a viral polymerase complex for its ability to perform both transcription and replication in cells. BHK cells were

infected with VVT7 and transfected in triplicate overnight with the plasmids encoding the MV N, P, and wt or mutant full length L proteins as well as one for the CAT mini-genome (p107CAT). These plasmids are all driven by the T7 promoter. The CAT mini-genome expresses a negative sense RNA which contains the CAT gene between authentic measles virus termini. This mini-genome is nonspecifically encapsidated by the MV N protein yielding a template for transcription and replication by the viral polymerase. The wt or mutant L proteins may or may not form a polymerase complex with the P protein. Only if a functional polymerase complex forms is there transcription and replication which leads to the expression of the CAT protein.

Cell extracts were prepared and incubated with chloramphenicol and ^{14}C-acetyl CoA as described in Materials and Methods. The CAT protein, if present, transfers the ^{14}C-acetyl group to chloramphenicol. The samples were then extracted with ethyl acetate and the organic layer was counted on a scintillation counter. The ^{14}C labeled acetylated chloramphenicol partitions to the organic layer, while the ^{14}C-acetyl CoA remains in the aqueous layer. The mock sample was transfected only with the CAT mini-genome which gave little or no background CAT activity from the template in the absence of MV proteins (Fig. 7A). Wt MV L showed good CAT activity, while all four of the mutant L

proteins gave no CAT activity (Fig. 7A). These data were consistent with the conclusion that the mutants MV L proteins were unable to form a polymerase complex. A portion of each triplicate cell extract was separated on an SDS-PAGE gel, transferred to a nylon membrane, and incubated with a rabbit α-MV antibody. The membrane was washed and then incubated with a goat α-rabbit secondary antibody and the proteins visualized by ECL as described in Materials and Methods. The mock transfected samples showed that there were no host or vaccinia virus background proteins detected by the antibody (Fig. 7B). The expression of the MV N and P protein is uniform in the each sample (Fig. 7B). Since we lack an antibody which can detect the wt or mutant MV L proteins in a Western we are unable to show the expression of the L protein by this method, although, radiolabeling experiments showed that each of the mutant proteins was expressed to some degree (Fig. 6A).

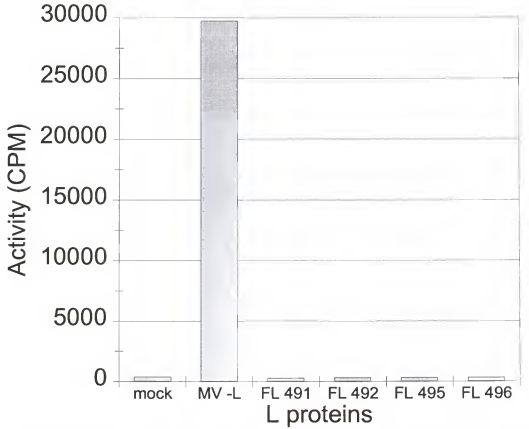
Sendai Virus

Site directed mutagenesis of the Sendai virus L protein to identify the P protein binding site

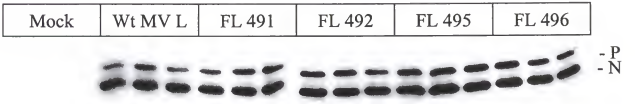
Previous experiments with carboxy-terminal truncation of SV L could not define a region smaller than aa 1-1147 for the P protein binding site. Based on the amino-terminal location of the P protein binding site on MV L, a panel of nine site directed mutants were created in the amino-

Figure 7. CAT assay testing the ability of wt and mutant measles virus polymerase complexes to perform in vitro transcription and replication in a mini-genome rescue system. BHK cells were infected by VVT7 at a m.o.i. of 2.5 and then transfected with 2.5 µg of p107CAT alone (mock) or 2.5 µg of p107CAT, 2.5 µg of pBSMV-NP, 5 µg pBSMV-P, and 0.5 µg of wt or mutant pBSMV-L. Cell extracts were prepared and CAT activity was measured as described in the materials and methods section. (**A**) Graphical representation of CAT activity, each bar represent the average value of an experiment done in triplicate. (**B**)Western assay was performed on sample of each experiment using α-MV antibody. The position of the protein bands are indicated on the right of the gel.

A


30000
25000
20000
15000
10000
5000
0
Activity (CPM)
mock
MV -L
FL 491
FL 492
FL 495
FL 496
L proteins

B


Mock
Wt MV L
FL 491
FL 492
FL 495
FL 496
- P
- N

terminal 400 aa of the wt Sendai virus L protein at locations corresponding to those in MV L (see Materials and Methods). Sendai virus provides a better system to study protein function since assays are available for many of the steps in viral RNA synthesis. The mutant plasmid DNAs were then tested for their ability to synthesize full length protein by translation in the TNT system. The protein products were analyzed by SDS-PAGE as detailed in Materials and Methods (Fig. 8). No protein synthesis was detected in the absence of added plasmid (Fig. 8, lane 1), while the wt SV L plasmid alone expressed a full length protein at relatively high levels (Fig. 8, lane 2). The faster migrating band is most likely a truncation of the full length protein. Mutants SV 518 and SV 519 were not expressed well possibly because they are not stable proteins (Fig. 8, lanes 7 and 8). The remaining L mutants were all able to synthesize full length protein products at levels comparable to wt SV L protein (Fig. 8, lanes 3-6, 9-11).

Polymerase complex formation of the SV L mutants

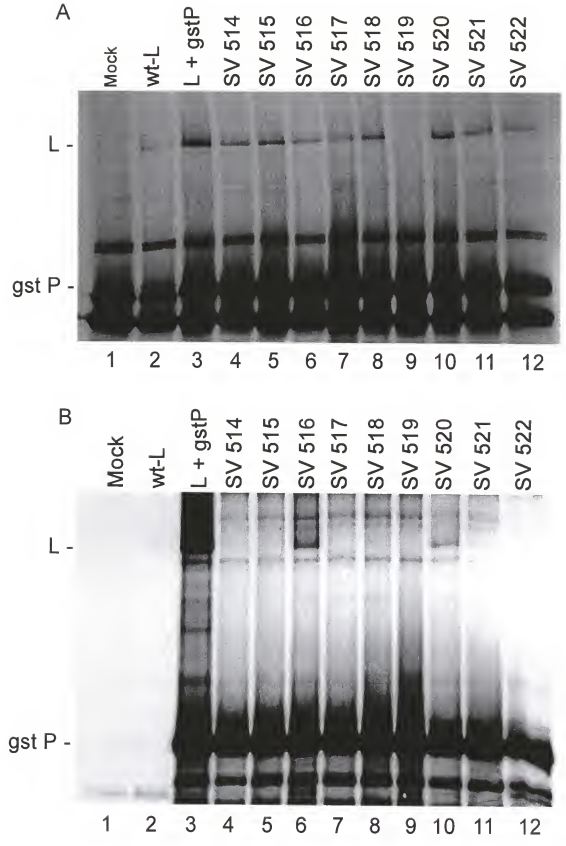
The ability of the SV L mutants to bind to the gstP protein and form a polymerase complex was tested in a mammalian expression system. A549 cells were infected with VVT7 to provide cytoplasmic expression of T7 RNA polymerase. The cells were then transfected with the SV gstP and the wt

Figure 8. Coupled transcription and translation of wt or mutant Sendai virus L plasmids using the TNT Coupled Reticulocyte Lysate System. T7 RNA polymerase, [^{3}H]-lysine, and 0.5 µg of wt or mutant SV L plasmids were incubated at 30^{0} C for 2 hrs with a rabbit reticulocyte lysate and the resultant protein product was visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein is indicated on the left of the gel.

L-
Mock
wt L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
1
2
3
4
5
6
7
8
9
10
11

or mutant SV L plasmids with each gene under the control of the T7 promoter. The cells were incubated with ^{35}S Express label overnight and cytoplasmic extracts were prepared as detailed in Materials and Methods. Samples of the extracts were analyzed directly for total protein expression (Fig. 9A). The mock transfected sample showed some background host and vaccinia virus proteins (Fig. 9A, lane 1). When wt SV L plasmid was expressed alone the L protein was barely detectable (Fig. 9A, lane 2) which is consistent with the instability of the SV L protein when not expressed with the SV P protein (Horikami et al., 1992). When wt SV L and SV gstP plasmids were co-expressed the gstP protein stabilizes the L protein with the result that both proteins are expressed well (Fig. 9A, lane 3). The mutant SV 519 protein gave no detectable L expression when co-expressed with the SV gstP protein (Fig. 9A, lane 9). Consistent with the lack of expression in the TNT system (Fig. 8), SV 519 appears unstable under any expression conditions. The other L mutants when co-expressed with the SV gstP protein gave detectable levels of L protein but they were all well below that of the wt SV L protein (Fig. 9A, lanes 4-8 and 10-12). SV 518 which was not expressed well in the TNT system, did show some expression in the mammalian expression system. Perhaps some component of the mammalian cell or co-expression with the SV gstP protein stabilized this L mutant

Figure 9. Total protein synthesis and bead binding assay to measure polymerase complex formation of the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 0.2 μg of SV gstP and 1.67 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were incubated with ^{35}S-EXPRESS for 18 hrs and then cytoplasmic extracts were prepared as detailed in the materials and methods section. The extract was then divided into two aliquots and (**A**) 10 μl of cell extract was run directly on a 7.5% SDS-PAGE gel to visualize total protein expression or (**B**) incubated with glutathione conjugated Sepharose beads and then washed. The bound proteins were visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein bands are indicated on the left of the gel.


A
Mock
wt-L
L + gstP
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
L -
gst P -
1 2 3 4 5 6 7 8 9 10 11 12
B
Mock
wt-L
L + gstP
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
L -
gst P -
1 2 3 4 5 6 7 8 9 10 11 12

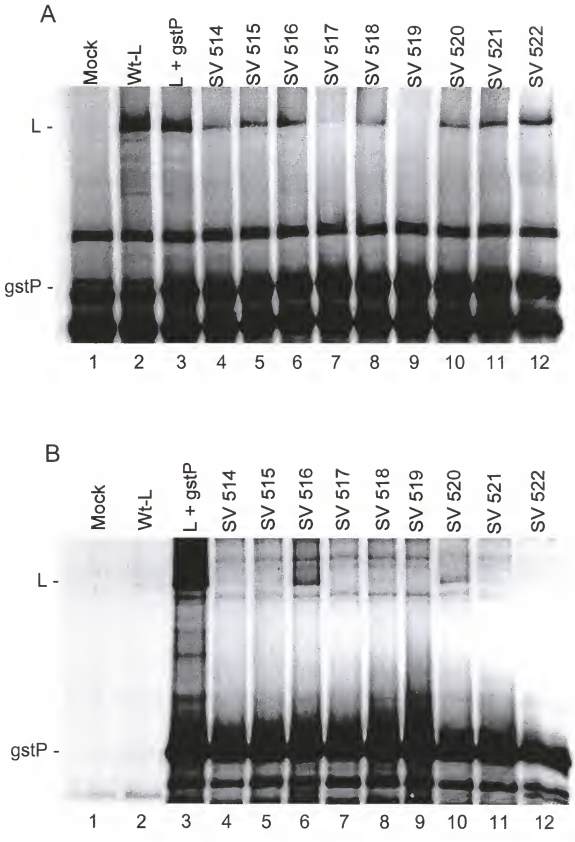
in vivo. The level of expression in these mutants resembled that of wt SV L alone, a first indication that these mutants may not be able to bind to SV gstP.

The mutant L proteins were tested for their ability to form a complex with the SV gstP protein in the glutathione conjugated Sepharose bead binding assay. The mock transfected sample showed that there were no background host or vaccinia virus proteins that non-specifically bound to the glutathione beads (Fig. 9B, lane 1). When wt SV L was expressed alone it was unable to bind to the beads, however, when the wt SV L and SV gstP proteins are co-expressed they both bind the beads (Fig. 9B, lanes 2 and 3). This is due to the ability of these two proteins to form a complex which binds to the beads via the gst portion of the gstP fusion protein. Previous experiments showed that the binding of the L protein is specific for the P portion of the gstP fusion protein (Horikami et al., 1992). The various L mutants were co-expressed with the SV gstP protein and tested for complex formation. Mutants SV 516 and SV 520 were both able to bind to some degree to the gstP fusion protein, but their expression was also much reduced when compared to wt SV L protein (Fig. 9 A and B, lanes 6 and 10). None of the remaining mutants were able to bind to SV gstP protein at detectable levels even after a long exposure of the gel (Fig. 9B, lanes 4, 5, 7, 8,and 10-12). These data show that 2 of the 9 mutants are capable of binding but

at levels much reduced as compared to the wt SV L protein and that 7 of the 9 mutants are completely unable to bind to the gstP protein, although the SV 519 mutant appears not to be made. Since the half life of wt SV L is only 1.2 hours when expressed alone it may be degrading almost as quickly as it is made. This could explain the lower levels of mutant SV L protein expression where binding did not occur the L proteins were degraded. Since the mutant L proteins are not present at anywhere near wt levels weak binding may not be detected.

In order to try to detect even low levels of P-L complex formation the experiments were repeated with short term labeling (30 min) of the infected and transfected cells. Analysis of total protein showed only the host and vaccinia virus background proteins in the mock transfected sample (Fig. 10A, lane 1). The wt SV L protein alone was expressed well in the short term label (Fig. 10A, lane 2) unlike in the long term labeling (Fig. 9A). When the wt SV L and SV gstP protein were co-expressed both were expressed well (Fig. 10A, lane 3). The level of expression of wt SV L alone (lane 2) actually slightly exceeded that of wt SV L in the co-transfection (lane 3). Since the cells are labeled for only 30 min which is shorter than the half life of the protein the difference between SV L alone and SV L stabilized with SV gstP diminishes. When wt SV L was transfected alone there was only one plasmid utilized by the

Figure 10. Total protein synthesis and bead binding assay to measure polymerase complex formation of the wt and mutant Sendai virus L proteins in a short term label experiment. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 0.2 μg of SV gstP and 1.67 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were transfected for 10 hrs and then the protein was labeled for 30 minutes using ^{35}S-EXPRESS. Cytoplasmic extracts were then prepared as detailed in the materials and methods section. The extract was then divided into two aliquots and (**A**) 10 μl of cell extract was run directly on a 7.5% SDS-PAGE gel to visualize total protein expression or (**B**) incubated with glutathione conjugated Sepharose beads and then washed. The bound proteins were visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein bands are indicated on the left of the gel.


A
Mock
Wt-L
L + gstP
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
L -
gstP -
1 2 3 4 5 6 7 8 9 10 11 12
B
Mock
Wt-L
L + gstP
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
L -
gstP -
1 2 3 4 5 6 7 8 9 10 11 12

translation machinery, while in lane 3 there are two plasmids which must compete for the translation machinery and this might lead to slightly less protein synthesis for each of the two plasmids. The various SV L mutants were co-expressed with the SV gstP protein as indicated above the gel (Fig. 10A). The mutant SV 517 protein expression levels were down compared to the other mutants indicating that this mutant is less stable most likely due to its inability to bind to the gstP protein. The mutant SV 519, as in the overnight labeling, was not detected even on a long exposure of the gel (Fig. 10A, lane 9). This suggests that the SV 519 mutant protein may be so unstable that it is degraded immediately in mammalian cells, although a small amount was detected by *in vitro* translation (Fig. 8). The remaining L mutants were still not synthesized as well as the wt SV L protein as observed in the overnight labeled total protein (Fig. 9A), suggesting that these proteins are intrinsically more unstable than wt SV L protein alone.

Analysis of the P-L complex formation gave similar results in the short term labeling similar to long term labeling. The mock transfected sample showed that no host or vaccinia virus proteins are able to bind to the glutathione beads (Fig. 10B, lane 1), nor did the wt SV L protein when expressed alone (Fig. 10B, lane 2). When SV gstP and wt SV L proteins are co-expressed complex formation occurs (Fig. 10B, lane 3), however, of the various SV L

mutants only the SV 516 and SV 520 proteins co-bound with the SV gstP protein to the beads (Fig. 10B, lanes 6 and 10). The remaining 7 mutants were all unable to bind to the SV gstP protein (Fig. 10B, lanes 4, 5, 7-9, 11, and 12) a result similar to that with long term labeling, indeed, these data suggest that 7 of the 9 SV L mutants are completely unable to form a complex with the SV gstP protein. Mutants SV 516 and SV 520 are able to bind SV gstP protein but at substantially reduced levels when compared with the wt SV L protein.

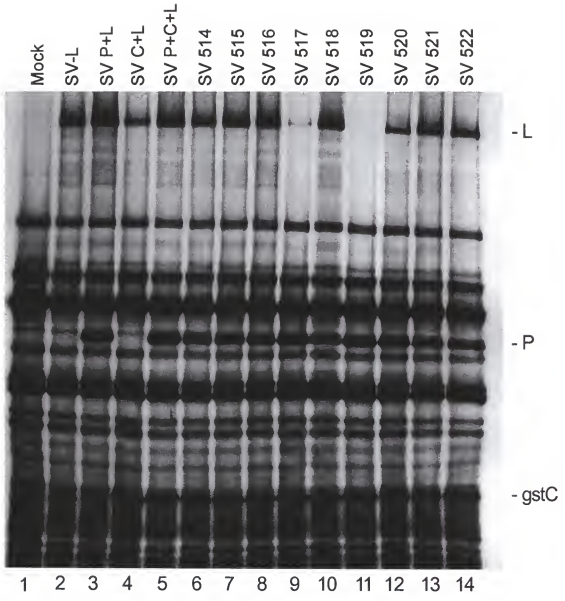
Mutant SV L protein interaction with the SV gstC protein

Since the mutant SV L proteins appeared inherently more unstable even in a short term label we tested if the mutations disrupted other functions of the L protein, by determining their ability to bind to the SV gstC protein. It is known that the gstC protein binds to the L protein at an unknown site which is distinct from the SV P protein binding site (Horikami et al., 1997). A549 cells were infected with VVT7 and transfected with the SV gstC plasmid together with SV P and the wt or mutant SV L plasmids. Analysis of total protein synthesis in a short term experiment by SDS-PAGE showed that the mock transfected sample had only the background host and vaccinia virus proteins (Fig. 11, lane 1). When wt SV L was expressed alone or with the P protein there was good expression of the

L protein (Fig. 11, lanes 2 and 3). When wt SV L and SV gstC protein were co-expressed in the absence or presence of the P protein the L protein was also expressed well (Fig. 11, lane 3 and 4). The SV P protein was expressed well in all lanes into which the P plasmid was transfected. The SV gstC protein comigrated with background host or vaccinia virus proteins which was detected as a slight increase of that band. The indicated mutant SV L proteins were then co-expressed with both the gstC and P proteins. The mutant SV 517 is barely detectable and does not appear to be stabilized by the co-expression of the gstC or P proteins (Fig. 11, lane 9). The mutant SV 519 still goes undetected even in a short term label experiment indicating that this protein is highly unstable when expressed in mammalian cells (Fig. 11, lane
11). The remaining L mutants were all synthesized with gstC at close to wt levels (Fig. 11, lanes 6-8, 10, and 12-14), in contrast to their expression with gstP alone (Fig. 10). This may be due to a stabilizing effect of the gstC protein when it binds to these mutant SV L proteins.

Analysis of L and P binding to the gstC protein was performed on a sample of the same cell extract. The mock transfected sample gave no binding to host or vaccinia virus background proteins as expected (Fig. 12, lane 1). Wt SV L alone or expressed with the P protein in the absence of gstC was unable to bind to the glutathione beads (Fig. 12, lane 2

Figure 11. Total protein synthesis of SV gstC and wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or with 0.2 μg SV gstC, 1.67 μg of SV Pstop and 1.67 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were incubated with ^{35}S-EXPRESS for 30 min and then cytoplasmic extracts were prepared as detailed in the materials and methods section. A 10 μl aliquot of cell extract was run directly on a 7.5% SDS-PAGE gel to visualize total protein expression by fluorography. The position of the protein bands are indicated on the left of the gel.

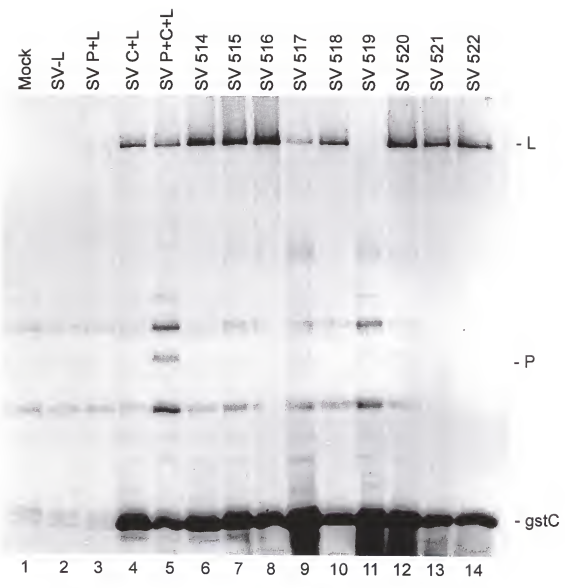

Mock
SV-L
SV P+L
SV C+L
SV P+C+L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
- L
- P
- gstC
1 2 3 4 5 6 7 8 9 10 11 12 13 14

and 3). When wt SV L and SV gstC proteins were co-expressed in either the absence or presence of P, the gstC fusion protein is able to form a complex with the SV L protein and bind to the glutathione beads (Fig. 12, lane 4 and 5). In the presence of the P protein the gstC-L complex bound P as described previously (Horikami et al., 1997). This confirms that the gstC protein binding site on the L protein is distinct from the P protein binding site. When the various mutant SV L proteins were co-expressed with both the P and gstC proteins only a small amount of the SV 517 protein is co-bound to the beads (Fig. 12, lane 9) and the mutant SV 519 protein was not detected at all (Fig. 12, lane 11). The remaining mutants were able to bind to gstC at close to or better than wt L levels (Fig. 12, lanes 6-8, 10, and 12-14). These data show that the C binding site on most of these mutant L proteins is intact which in turn suggest that the overall structure of these proteins is not disrupted. These mutations, therefore, specifically knock out the P binding site on the L protein.

Activity of the mutant SV L proteins in *in vitro* transcription

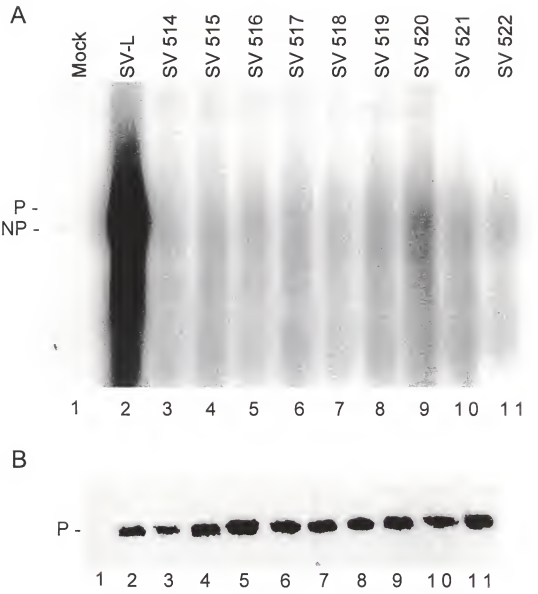
The mutant SV L proteins were then tested for their ability to synthesize the various viral RNAs *in vitro* and *in vivo*. First they were tested for their ability to perform transcription *in vitro* as described in Materials and Methods. We would predict that the L proteins which could

Figure 12. Bead binding assay to measure complex formation between SV gstC protein and the wt or mutant polymerase complex. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or with .02 μg SV gstC, 1.67 μg of SV Pstop and 1.67 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were incubated with ^{35}S-EXPRESS for 30 min and then cytoplasmic extracts were prepared as detailed in the materials and methods section. The expressed proteins were incubated with glutathione conjugated Sepharose beads and then washed. The bound proteins were visualized by fluorography on a 7.5% SDS-PAGE gel. The position of the protein bands are indicated on the left of the gel.


Mock
SV-L
SV P+L
SV C+L
SV P+C+L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
- L
- P
- gstC
1 2 3 4 5 6 7 8 9 10 11 12 13 14

not bind to the gstP protein will be inactive in all RNA synthesis. A549 cells were infected with VVT7, transfected with the SV P and wt or mutant SV L plasmids and cell extracts were prepared as described in Materials and Methods. The extracts were incubated with wt polymerase-free template in the presence of [α-^{32}P]CTP and the mRNA products were analyzed by gel electrophoresis. Previous data showed that the NP and P transcripts are both produced but they are approximately the same size and are not distinguishable in this gel system. No mRNA product was detected from the template in the absence of viral proteins (Fig. 13A, lane 1). As the positive control the wt SV L and SV P proteins were co-expressed and they gave strong transcription (Fig. 13A, lane 2). None of the SV L mutant proteins co-expressed with the SV P protein gave mRNA synthesis except for the SV 520 mutant protein which showed 7% of the activity of wt SV L protein (Fig. 13A). This is interesting because while both SV 516 and SV 520 gave binding to the SV gstP protein (Figs. 9B and 10B) only SV 520 was able to give some activity. Western analysis of samples of the extracts showed that the P protein expression was similar in all of the samples (Fig. 13B). Thus the lack of binding to the P protein correlated with the lack of function for the majority of the mutants.

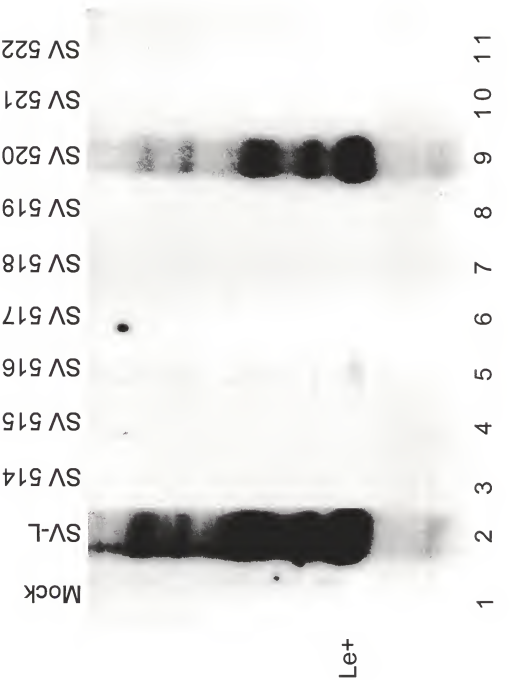
Figure 13. *in vitro* transcription with the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 1.5 μg of SV Pstop and 0.5 μg of wt or mutant SV L plasmids as indicated above each lane. (**A**) The cells were incubated at 37^0 C for 18 hrs and then cytoplasmic extracts were prepared. The extracts were incubated with polymerase free wt SV nucleocapsid template and [α-^{32}P]CTP. Total RNA was isolated using the Qiagen Rneasy kit and the separated on an agarose-urea gel as described in the materials and methods section. The position of the NP and P transcript is indicated to the left of the gel. (**B**) Samples of the cell extract were subjected to Western blot analysis using α-P antibody. The position of the P protein is indicated to the left of the gel.


A
Mock
SV-L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
P -
NP -
1 2 3 4 5 6 7 8 9 10 11
B
P -
1 2 3 4 5 6 7 8 9 10 11

Activity of the mutant SV L proteins in *in vitro* leader RNA synthesis

The first product of transcription is actually the small 55 nt leader RNA and not the NP mRNA. In order to study (+) strand leader RNA synthesis VVT7 infected A549 cells were transfected with the SV P and wt or mutant SV L proteins and cell extracts were prepared as described in Materials and Methods. The extracts were incubated with wt polymerase-free template and the unlabeled RNA products were separated on a polyacrylamide-urea gel. The RNA was then transferred to a membrane which was then probed using an end-labeled oligo specific for the (+) leader RNA as detailed in Materials and Methods. The mock transfected sample gave no RNA synthesis (Fig. 14, lane 1), however, when the wt L and P proteins are co-expressed they gave a strong leader signal (Fig. 14, lane 2). The longer products are most likely read through transcripts by the polymerase complex. The majority of the L mutant proteins were unable to synthesize any detectable leader RNA products (Fig. 14, lanes 3, 4, 6-8, 10, and 11). However, the mutant SV 516 protein had approximately 4% of the activity of the wt polymerase complex (Fig. 14, lane 5), whereas it was unable to give any detectable mRNA synthesis. The mutant SV 520 protein gave very good leader RNA synthesis which was about 60% of the activity of the wt polymerase complex (Fig. 14, lane 9), while mRNA synthesis (7%) was considerably less

Figure 14. *in vitro* Leader RNA synthesis with the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 1.5 μg of SV Pstop and 0.5 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were incubated at 37^0 C for 18 hrs and then cytoplasmic extracts were prepared. The extracts were incubated with polymerase free wt SV nucleocapsid template and the leader RNA was detect by Northern analysis using a probe complementary to the leader RNA as described in the materials and methods section. The 55 nt leader product is indicated to the left of the gel.

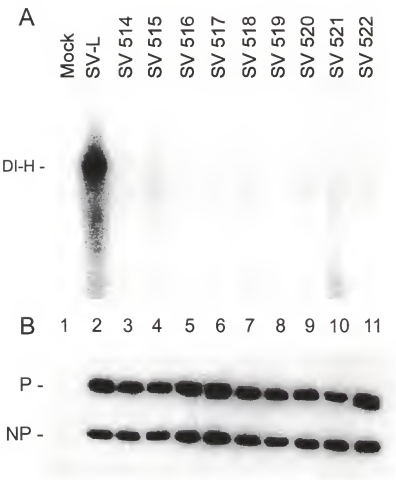

Le+
1 2 3 4 5 6 7 8 9 10 11
Mock
SV-L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522

(Fig. 13, lane 9). This activity in transcription is consistent with the finding that both SV 516 and SV 520 gave some SV gstP binding in both the long and short term labeled bead binding experiments (Figs. 9 and 10).

Activity of the mutant SV L proteins in *in vitro* replication

The mutants were next tested for their *in vitro* replication activity. This assay measures the ability of the polymerase complex to synthesize genomic length DI RNA that is encapsidated by the NP protein and as such is nuclease resistant. VVT7 infected A549 cells were transfected overnight with either no plasmid (mock) or with the SV NP, P, and wt or mutant L plasmids. Cell extracts were prepared and incubated with DI-H polymerase-free template and [α-^{32}P]CTP as detailed in Materials and Methods. Nuclease resistant, encapsidated RNA products were isolated and analyzed on an agarose-urea gel. No *in vitro* replication activity was observed in the mock transfected extract, while with co-expression of the SV NP, SV P, and wt SV L proteins a strong replication product results (Fig. 15A, lanes 1 and 2). When the various SV L mutant proteins were co-expressed with the SV NP and P proteins none gave any *in vitro* replication products (Fig. 15A, lanes 3-11). Western analysis of samples of the extract showed that the NP and P proteins were uniformly expressed (Fig. 15B). For the seven mutants that are unable to form a polymerase

Figure 15. *in vitro* DI-H Replication with the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 2 μg NP, 5 μg of Pstop and 0.5 μg of wt or mutant SV L plasmids as indicated above each lane. Cytoplasmic extracts were prepared and then incubated with DI-H nucleocapsid template in the presence of [α-^{32}P]CTP for 2 hrs. The extracts were then nuclease treated and the nuclease resistant RNA was isolated using the Qiagen Rneasy kit and the separated on an agarose-urea gel as described in the materials and methods section. The position of the DI-H RNA is indicated to the left of the gel. (**B**) Samples of the cell extracts were subjected to Western blot analysis using α-SV and α-P antibodies. The position of the NP and P proteins are indicated to the left of the gel.


A
Mock
SV-L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
DI-H -
B
1 2 3 4 5 6 7 8 9 10 11
P -
NP -

complex this can be explained by the fact that formation of the polymerase complex is required to give replication. The mutant SV 516 and SV 520 proteins were able to give a small amount of complex formation but these complexes were unable to catalyze *in vitro* replication, although they were able to give some transcription.

Activity of the mutant SV L proteins in *in vivo* replication

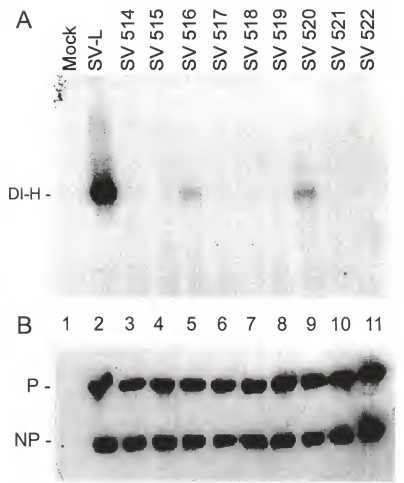
Next the wt and mutants SV L proteins were tested for their ability to perform *in vivo* replication. A549 cells were infected by VVT7 in order to provide cytoplasmic expression of the T7 RNA polymerase. The cells were then transfected overnight with the SV NP, P, and wt or mutant SV L plasmids in addition to the pSPDI-H plasmid which encodes a (+) strand DI-H defective interfering genome RNA. The pSPDI-H plasmid transcribes the DI-H mini-genome which is nonspecifically encapsidated by the NP protein. Once encapsidated the mini-genome becomes a template for replication by the viral polymerase complex in cells. Cell extracts were prepared, nuclease resistant RNA was extracted, separated on an agarose-urea gel, and then transferred to a nitrocellulose membrane. The (-) sense replication products were detected by Northern analysis with a (+) sense DI riboprobe. No replication products were detected in the mock transfected extract (Fig. 16A, lane 1) the absence of viral proteins. This shows that there was no

background activity from the DI plasmid in A549 cells. The wt polymerase complex gave good DI replication (Fig. 16A, lane 2). The majority of mutant SV L proteins gave no *in vivo* replication activity (Fig. 16A, lanes 3, 4, 6-8, 10, and 11). Again only mutants SV 516 and SV 520 gave any *in vivo* replication product with 6% and 11% activity as compared to the wt SV L protein, respectively (Fig. 16A, lanes 5 and 9). Western blot analysis showed that the NP and P proteins were uniformly synthesized (Fig. 16B). Mutants which were not able to give an *in vivo* replication product were also not able to form a polymerase complex, whereas, the mutants able to give some binding were able to give limited transcription and *in vivo* but not *in vitro* replication activity.

Binding of the mutant SV L protein to nucleocapsids

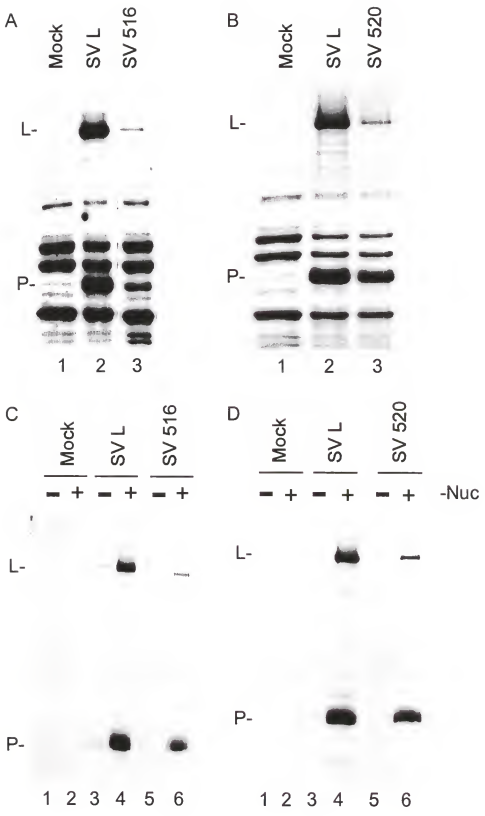
The L protein is unable to bind to the nucleocapsid template on its own, therefore, one of the functions of the P protein is to bind the P-L polymerase complex to the nucleocapsid template. The P protein has a supplemental function in which it is thought to help the polymerase complex see the encapsidated RNA, so unlike the L protein the P protein can bind to the nucleocapsid template by itself. The data presented here has shown that 7 of the 9 mutant L proteins were unable to bind to the gstP fusion protein and form a complex and were inactive in all RNA

Figure 16. *in vivo* Replication with the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 2.5 μg pSPDI-H, 2 μg NP, 5 μg of Pstop and 0.5 μg of wt or mutant SV L plasmids as indicated above each lane. Following an 18 hr incubation cytoplasmic extracts were prepared as detailed in Materials and Methods and treated with micrococcal nuclease. The nuclease resistant RNA was isolated, separated on an agarose-urea gel, and then analyzed by Northern analysis. The blot was probed with a DI-H specifiv (+) sense ^{32}P labeled riboprobe as described in Materials and Methods. (**B**) Samples of the cell extracts were subjected to Western blot analysis using α-SV and α-P antibodies. The position of the DI-H RNA and proteins are indicated to the left of the gel.


A
Mock
SV-L
SV 514
SV 515
SV 516
SV 517
SV 518
SV 519
SV 520
SV 521
SV 522
DI-H -
B
1 2 3 4 5 6 7 8 9 10 11
P -
NP -

synthesis. Another method to investigate P-L polymerase complex formation that does not rely on a fusion protein is to measure the binding of P-L complexes to the nucleocapsid template by a co-sedimentation assay. We would expect P to bind and for L to bind as well only if the P and L proteins form a complex. The two mutant L proteins capable of some binding to the gstP protein, SV 516 and SV 520, were tested for their ability to form a polymerase complex capable of binding to nucleocapsids *in vitro*. A549 cells were infected with VVT7, transfected with the SV P and the wt or mutant SV L plasmids and then they were labeled with ^{35}S Express overnight. Cytoplasmic extracts were prepared and analyzed for total protein synthesis by SDS-PAGE (Fig. 17 A + B). The mock transfected sample showed only the host and vaccinia virus background proteins (Figs. 17 A + B, lanes 1). When wt L and P proteins are co-expressed they are both expressed well (Figs. 17 A + B, lanes 2). The SV 516 (Fig. 17A, lane 3) and SV 520 (Fig. 17B, lane 3) showed some protein synthesis, although not at wt levels as observed previously. Samples of the cell extracts were incubated with and without polymerase-free nucleocapsids. This allows any polymerase complex which maybe formed to associate with the nucleocapsid. The extracts were layered over a glycerol gradient and then the nucleocapsids were pelleted in an ultracentifuge. The pellets were resuspended and then analyzed on a SDS-PAGE gel. The mock transfected extract

Figure 17. Nucleocapsid Binding assay *in vitro* with the wt or mutant Sendai virus L proteins. A549 cells were infected by VVT7 at m.o.i. of 2.5 and then transfected with no plasmids (mock) or 15 μg of Pstop and 15 μg of wt or mutant SV L plasmids as indicated above each lane. The cells were incubated overnight with ^{35}S-EXPRESS at 37^{0} C and extracts were performed as described in the materials and methods section. (**A + B**) 10 μl of cell extract was run on a 7.5 % SDS-PAGE gel to visualize total protein. (**C + D**) Cell extracts were incubated in the absence (-) and presence (+) of wt SV polymerase free nucleocapsids and then pelleted through glycerol by ultracentrifugation as described in the materials and methods section. The proteins associated with the nucleocapsids were analyzed on a 7.5% SDS-PAGE gel and visualized by fluorography. The position of the P and L proteins are indicated to the left of the gel.


A
Mock
SV L
SV 516
L-
P-
1 2 3
B
Mock
SV L
SV 520
L-
P-
1 2 3
C
Mock
SV L
SV 516
- + - + - +
L-
P-
1 2 3 4 5 6
D
Mock
SV L
SV 520
- + - + - +
-Nuc
L-
P-
1 2 3 4 5 6

both with and without nucleocapsids gave no host or vaccinia virus background proteins when pelleted in an ultracentrifuge (Figs. 17 C + D, lanes 1 and 2). When wt SV L and P proteins were co-expressed in the absence of nucleocapsids (-), small amounts of P and L pelleted (Figs. 17 C + D, lanes 3). This is most likely aggregated product that sediments upon centrifugation. The presence of nucleocapsids (+) greatly increased the amounts of the P protein bound to the nucleocapsid template and the presence of wt L as well showed complex formation (Figs. 17 C + D, lanes 4). Without nucleocapsids the L mutants failed to pellet (Figs. 17C + D, lanes 5). In the presence of nucleocapsids the mutant SV 516 and SV 520 proteins co-bound with the SV P protein and formed a polymerase complex capable of binding nucleocapsids *in vitro* (Figs. 17 A + B, lanes 6). These data are consistent with the bead binding which showed that these two mutants were able to form at least some polymerase complex.

CHAPTER 4
DISCUSSION

Discussion

The purpose of this work is to locate the P protein binding site on the L protein of both Sendai and measles viruses. Previous work in this area utilized truncations of the Sendai, measles, and SV-5 virus L proteins and identified rather large portions of the L proteins as required for binding to their respective P proteins. Truncation and deletion analysis of the SV-5 L protein showed that the P binding site resides within the amino-terminal 1247 aa of the SV-5 L protein (Parks, 1994b). The measles and Sendai virus L proteins were tested for their ability to form complexes with their homologous P proteins. The Sendai virus L protein could be truncated to the amino-terminal 1147 aa and still retained the ability to bind to the P protein (Horikami et al., 1997). A shorter truncation to the amino-terminal 875 aa of the SV L protein gave a protein which was unable to form this complex. In measles virus the L protein could be truncated to the amino-terminal 408 aa and still retained P protein binding (Chandrika et al., 1995a).

Twelve site-directed mutants were constructed in the truncated MV 408 protein in order to determine if P protein binding could be eliminated (Fig. 18). The mutagenesis strategy was changing clustered hydrophobic or charged amino acids to alanine in the hopes of knocking out aa important for the P-L protein-protein interaction. The mutants were predominantly hydrophobic to alanine changes with the exceptions of the following four mutants: MV 492, MV 494, MV 502, and MV 508. The twelve mutant proteins were all synthesized as well as the wt MV 408 protein in the *in vitro* coupled transcription and translation system (Fig. 4). All of the mutants were sequenced and shown to encode a protein of the correct size, however, several had aberrant mobilities as compared to wt MV 408 protein. This is most likely due to alterations in SDS binding due to the amino acid changes. When the wt and mutant 408 proteins were tested for their ability to form a complex with the MV gstP protein it was found that four of the mutants (MV 494, MV 501, MV 502, and MV 508) retained gstP binding at close to wt levels, one namely MV 491, gave complex formation at the very limit of detection, and the remaining seven mutants gave no detectable complex formation (Fig. 5). This is a strong indication that the P binding site on the L protein resides within the amino-terminal 408 aa for measles virus.

Figure 18. Primary amino acid sequence of MV 408 protein. The amino acids changed to alanine are indicated in bold and are underlined. The amino acid changed from the measles virus to the Sendai virus amino acid and are indicated in blue and are underlined. The name of the mutation created by the amino acid changes are indicated in bold below the underline.

MDSLSVNQIL YPEVHLDSPI VTNKIVAILE YARVPHAYSL EDPTLCQNIK

MV 491

HRLKNGFSNQ MIINNVEVGN VIKSKLRSYP AHSHIPYPNC NQDLFNIEDK

MV 492

ESTRKIRELL KKGNSLYSKV SDKVFQCLRD TNSRLGLGSE LREDIKEKVI

MV 494

NLGVYMHSSQ WFEPFLFWFT VKTEMRSVIK SQTHTCHRRR HTPVFFTGSS

MV 495

VELLISRDLV AIISKESQHV YYLTFEVLLI YCDVIEGRLM TETAMTIDAR

MV 496 MV 497

YTELLGRVRY MWKLIDGFFP ALGNPTYQIV AMLEPLSLAY LQLRDITVEL

MV 498 MV 499

RGAFLNHCFT EIHDVLDQNG FSDEGTYHEL IEALDYIFIT DDIHLTGEIR

MV 500 MV 501

SFFRSFGHPS LEAVTAAENV RKYMNQPKVI VYETLMKGHA IFCGIIINGY

MV 508 MV 502

Four of the MV 408 mutants which were unable to form a complex with the gstP protein were subcloned into the full in the context of the intact, full length L protein. It is possible that there are multiple domains within the L protein which are important for the binding to the P protein. With the mutations subcloned into the full length L protein one might detect other regions that are important for P protein binding. The mutant full length L proteins are all synthesized but their expression levels were low when compared with the wt MV L protein (Fig. 6). This is interesting because the mutant L proteins were all co-expressed with the gstP protein, but their expression levels were more similar to wt SV L protein when it was expressed alone. It is known that in Sendai virus the L protein is stabilized when co-expressed with the gstP protein. The fact that the mutant proteins resemble wt MV L without the gstP protein is suggestive that these mutants are not binding to gstP and as such their expression is not stabilized. This is supported by the bead binding assay which detects gstP and L protein-protein interactions where, with the exception of FL 491, none of the other mutants gave detectable gstP-L complex formation (Fig. 6). The mutant FL 491 protein gave only a small amount of complex formation which was at the limit of detection in this assay. This result correlates well with the same mutation in the

truncated MV 408 where MV 491 also gave barely detectable complex formation. These data suggest that the P binding site is restricted to the amino-terminal portion of the L protein. If another region of the L protein was important for P binding it might be expected that these mutants would give some gstP binding in the full length mutants.

One of the hypotheses of my project is that P-L complex formation is critical for the formation of an active polymerase. In order to test for biological activity the wt and mutant full length measles virus L proteins were assayed in cells for total RNA synthesis using a mini-genome CAT reporter system. This CAT mini-genome was rescued by co-expression with the N, P and wt or mutant L proteins and the results showed that only the wt L protein was able to form a polymerase complex capable of performing the transcriptive and replicative functions measured by CAT activity (Fig. 7). The FL 491 mutant protein which formed a small amount of the polymerase complex gave no CAT activity, indicating that the complex concentration in this case was either to low to measure or that the complex was defective in some required function.

The measles virus data showed that mutants of the amino-terminal aa of the MV L protein were defective in polymerase complex formation and that these mutants were also not competent for *in vitro* RNA synthesis. Analysis of

the types of mutations which eliminated the ability of the L protein to bind to the P protein indicate that of the four charge to alanine mutants only MV 492 eliminated binding to the P protein. In contrast of the eight hydrophobic to alanine mutants only the mutant MV 501 was still able to form a polymerase complex. This is suggestive that the P-L protein to protein interface in measles virus may be mainly due to hydrophobic interactions.

In Sendai virus we and others have developed *in vitro* assays for many of the individual steps of RNA synthesis unlike in measles virus. Assuming a homology in the location of the P binding domain in the L proteins of these two viruses site-directed mutagenesis was performed on the amino-terminus of the SV L protein. In Sendai virus the mutagenesis strategy we adopted was a measles to Sendai virus strategy. Many of the SV L mutants are changed to the sequences present in the MV 408 mutants. The remaining mutants were created using a clustered hydrophobic or charged amino acid to alanine strategy (Fig. 19). In contrast with measles virus, we constructed all of the mutations in the full length Sendai virus L protein so that they may be tested in the various functional assays available in Sendai virus. By *in vitro* translation the wt and mutant SV L proteins were all able to synthesize full length protein, however, mutants SV 518 and SV 519 expressed

Figure 19. Primary sequence of the amino-terminal 400 amino acids of the Sendai virus L protein. The amino acids changed to alanine are indicated in bold and are underlined. The amino acids changed from the Sendai virus to the measles virus amino acid and are indicated in blue and are underlined. The name of the mutation created by the amino acid changes are indicated in bold below the underline.

MDGQESSQNP SDILYPECHL NSPIVRGKTA QLHVLLDVNQ PYRLKDDSII

SV 514 SV 515

NITKHKIRNG GLSPRQIKIR SLGKALQRTI KDLDRYTFEP YPTYSQELLR

SV 516

LDIPEICDKI RSVFAVSDRL TRELSSGFQD LWLNIFKQLG NIEGREGYDP

LQDIGTIPEI TDKYSRNRWY RPFLTWFSIK YDMRWMQKTR PGGPLDTSNS

SV 517

HNLLECKSYE TVTYGDLVMI LNKLTLTGYI LTPELVLMYC DVVEGRWNMS

SV 518 SV 519

AAGHLDKKSI GLLGRGEELW ELVDSLFSSL GEEIYNVILL AEPLSLALIQ

SV 520 SV 521

LNDPVIPLRG AFMRHVLTEL QTVLTSRDVY TDAEADTIVE SLLAIFHGTS

SV 522

much less L protein as compared to the wt L protein (Fig. 8). The introduced mutations may disrupt the structure of these latter L proteins such that they are highly unstable. The remaining seven mutants all synthesized full length L protein at wt levels. When expressed in mammalian cells with the SV P protein and labeled overnight the wt and mutant SV L protein with the exception of SV 519 all gave full length protein expression. The levels of expression of the mutants resembled the wt L protein when expressed alone. Like the results with measles virus this suggests that the mutants are not binding to the P protein and as such are not stabilized by co-expression. In a direct assay for binding the wt SV L protein was able to form a good complex with the gstP protein, while only two of the nine mutants, namely SV 516 and SV 520, gave a small amount of complex. The remaining seven mutants all failed to generate any detectable gstP-L complex formation (Fig. 9). These seven mutants and even the two that gave some complex formation apparently are not stabilized by co-expression of the gstP protein. If some of these seven non-binding mutants are binding extremely weakly they may not be detected by radio-labeling overnight, so the experiment was repeated with a pulse labeling where the L proteins with short half lives which can still bind to the gstP protein might be detected. Again the wt and mutant SV L proteins all expressed full

length protein except for the mutant SV 519 protein. The low level of expression of the mutant proteins were not significantly different from the overnight labeling, although wt L alone increased (Fig. 10). Again only the mutants SV 516 and SV 520 gave any complex formation, although it was much reduced when compared to the wt SV L protein (Fig. 10). Therefore, it is likely that the seven mutants which do not give detectable complex formation are completely unable to bind to the P protein.

There are at least two explanations for why the mutant L proteins are unable to bind to the P protein. First the structures of these mutant L proteins maybe totally disrupted and the proteins are degraded as quickly as they are synthesized for instance the SV 519 protein. Alternatively, the P protein binding site on L maybe disrupted such that binding can not occur and the L protein is unstable as a result. In order to differentiate between these two possibilities the wt and mutant SV L proteins were tested for their ability to bind to a different protein, the C protein (Fig. 11). It is known that the C protein binds to the polymerase via the L protein and that the location of the C protein binding site on the L protein, although unknown, is distinct from that of the P protein. If the mutants which are unable to give any P-L complex formation are able to bind to the gstC protein in a short term

labeling experiment it would argue that the overall structure of the L protein is at least partially intact. In contrast to the former results, with gstC the mutant L proteins were all detected at close to wt levels, with the exception of the mutant SV 517 and SV 519 proteins (Fig. 10). The SV 517 protein expression was low suggesting that the gstC protein is not stabilizing this mutant protein as well as the other mutants, although it still clearly bound some of the gstC protein (Fig. 11). The SV 519 protein is apparently completely unstable for its expression was never detected regardless of the presence of other viral proteins. Furthermore all of the mutants except for the SV 519 protein were capable of binding to the gstC protein, suggesting that the structure of most of the mutant SV L proteins was not entirely disrupted. These are encouraging results because mutants which are deficient in P protein binding but intact otherwise are desirable so that we can test if complex formation is required for polymerase function.

Up to this point all tests for protein-protein interactions have been done with gst fusion proteins. This is a practical way to test for complex formation but it is possible that the gst moiety in some cases may be interfering with the P-L interaction. A method which gets around this problem is to co-express wt or mutant SV L protein with the wt P protein and then test if the

polymerase complex can bind to nucleocapsids. Of the various mutant SV L proteins only the SV 516 and SV 520 are able to form a complex with the gstP protein. These two mutants were co-expressed with the P protein and they were both able to form a polymerase complex which co-sediments with the nucleocapsids.

The mutant SV L proteins were then tested for their ability to perform the various steps of RNA synthesis. The SV 516 and SV 520 proteins were the only mutant L proteins able to give any RNA synthesis, while the remaining seven mutants were not able to give any detectable RNA synthesis (Table 6). This correlates well with the gstP bead binding and nucleocapsid binding data in that only mutants capable of some complex formation can give any RNA synthesis.

The SV 516 protein though capable of forming a weak complex with the gstP protein was unable to give any *in vitro* mRNA synthesis (Table 6). This shows that complex formation while required is not sufficient for *in vitro* transcription activity. The SV 516 protein was capable of giving a small amount of leader RNA synthesis which was just 4% of the wt polymerase complex (Table 6). This mutant was capable of getting to the nucleocapsids, although it is much reduced when compared to the wt polymerase complex, but its ability to catalyze leader RNA was severely inhibited.

Table 6. Summary of the protein-protein interaction and RNA synthesis data for Sendai virus site-directed L mutants

	Protein Binding			Transcription (%)		Replication (%)	
L mutant	gstP	gstC	NC	mRNA	le+ RNA	In vitro	In vivo
wt	++++	++++	++++	100	100	100	100
SV 514	-	++++	-	0	0	0	0
SV 515	-	++++	-	0	0	0	0
SV 516	+	++++	+	0	4	0	6
SV 517	-	+	-	0	0	0	0
SV 518	-	++++	-	0	0	0	0
SV 519	-	-	-	0	0	0	0
SV 520	++	++++	++	7	62	0	11
SV 521	-	++++	-	0	0	0	0
SV 522	-	++++	-	0	0	0	0

The plus sign under protein binding refer to the amount of protein-protein interaction as compared to wild type L, where ++++ indicates 100% binding, ++ indicates 20-30%, and + indicates <20%. The numbers under transcription and replication indicate percent of wt polymerase complex function, which is arbitrarily set at 100%.

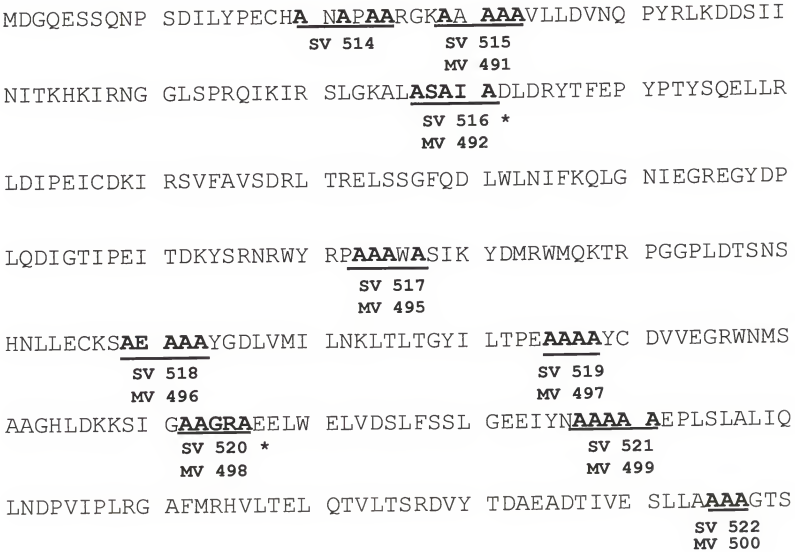
Since the polymerase complex fails to reinitiate ~30% of the time at the gene junction based on the level of leader RNA synthesis it is not surprising that no mRNA synthesis was detected. The SV 516 mutant protein was unable to give any *in vitro* replication, but was able to give ~6% *in vivo* replication as compared to the wt polymerase complex (Table 6).

The SV 520 protein was the only mutant capable of giving *in vitro* transcription with an activity that was just 7% of the wt polymerase complex (Table 6). The SV 520 protein, however, gave good leader RNA synthesis which was 62% of the wt polymerase complex (Table 6). Based on the attenuation of transcription with the wt polymerase complex with leader RNA synthesis at 62% of wt one would expect mRNA synthesis of around 43% of wt L. One way to explain this result is that the mutant polymerase complex gets to the nucleocapsids efficiently and it is able to synthesize the leader RNA but is unable to get beyond the first gene junction and reinitiate mRNA synthesis downstream of leader. This mutant polymerase complex may well be stuck on the leader gene where it continues to reiteratively synthesize leader RNA but only rarely moves past the gene junction to give mRNA synthesis at the NP start site. The SV 520 protein was unable to synthesize any *in vitro* replication products but was able to give ~11% *in vivo* replication as

compared to the wt polymerase complex (Table 6). The difference in the two replication experiments is most likely due to the fact that *in vivo* replication takes place in intact cells and it maybe that replication is more efficient when it associates with some cellular structure disrupted in the cell extracts used for *in vitro* replication. Clearly the wt polymerase complex is capable of performing *in vitro* replication. It may be that there is a component within intact cells that confers an increased efficiency specifically for this mutant.

In measles virus the type of mutation which most affected binding to gstP were the hydrophobic to alanine. In fact of the seven MV 408 mutants which are unable to bind to gstP six used the hydrophobic to alanine strategy. In Sendai virus the result is the same of the seven mutants unable to bind to the P protein six were created using the hydrophobic to alanine mutagenesis strategy. The Sendai mutants were created, except for SV 514, at the same locations as the measles virus mutants. In general the phenotype of the mutants is consistent in both measles and Sendai viruses but mutants SV 516 and SV 520 gave a some binding while their measles virus correlates did not (Fig. 20). This result indicates that the interaction between the P and L proteins is hydrophobic in nature. Sequence alignment of the amino-terminal 408 aa of measles and Sendai

Figure 20. Primary sequence of the amino-terminal 400 amino acids of the Sendai virus L protein. The amino acids changed are indicated in bold and are underlined with the name of the Sendai virus mutant above its measles virus correlate. The asterisk (*) indicates the two mutants in Sendai virus capable of complex formation with gstP and of binding to nucleocapsids.


MDGQESSQNP SDILYPECHA NAPAARGKAA AAAVLLDVNQ PYRLKDDSII
SV 514
SV 515
MV 491
NITKHKIRNG GLSPRQIKIR SLGKALASAI ADLDRYTFEP YPTYSQELLR
SV 516 *
MV 492
LDIPEICDKI RSVFAVSDRL TRELSSGFQD LWLNIFKQLG NIEGREGYDP
LQDIGTIPEI TDKYSRNRWY RPAAAWASIK YDMRWMQKTR PGGPLDTSNS
SV 517
MV 495
HNLLECKSAE AAAYGDLVMI LNKLTLTGYI LTPEAAAAYC DVVEGRWNMS
SV 518
MV 496
SV 519
MV 497
AAGHLDKKSI GAAGRAEELW ELVDSLFSSL GEEIYNAAAA AEPLSLALIQ
SV 520 *
MV 498
SV 521
MV 499
LNDPVIPLRG AFMRHVLTEL QTVLTSRDVY TDAEADTIVE SLLAAAAGTS
SV 522
MV 500

virus show that aa conservation in this region of the protein is relatively low (Fig. 21). The location of the Sendai virus mutant is shown to indicate that most of the mutants were targeted to regions of little or no conservation with the exception of the mutant SV 514, SV 517, and SV 519 proteins. The mutant SV 517 and SV 519 protein were not synthesized to detectable levels in the gstP bead binding assay indicating that these mutation may target structural components of the P binding site on the SV L protein and as such are to unstable to be expressed.

Protein synthesis in all protein-protein interaction experiments was detected by radiolabeling with ^{35}S Translabel. The weak signal of ^{35}S makes quantitation of protein expression using a phosphoimager unreliable and as such determination of relative levels of protein expression are based on visual estimations. Based on these limitations we estimate that approximately 50-60% of the total SV wt L protein expressed forms a detectable complex with the gstP protein. This can be seen by comparing wt L expression in totals compared with the amount of L protein brought down in the bead binding assay (Figs. 9 and 10). With the exception of the mutant SV 517 and SV 519 proteins, which were never detected in any gstP bead binding experiment, all of the mutants were synthesized at approximately 20% or lower when compared with the wt L protein when co-expressed with the

Figure 21. Sequence alignment of the amino-terminal 408 amino acids of the measles and Sendai virus L proteins. The first line of the alignment indicates the amino-terminal amino acids of the measles virus L protein. The second line of the alignment indicates the amino-terminal amino acids of the Sendai virus L protein. The third line indicates only the conserved amino acids of the alignment. The Sendai virus mutation sites are indicated above the sequence alignment. The amino acid highlighted in grey with the black text indicate location of conserved changes between the two viruses and the amino acid in red text indicate location of conservation.

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                        1                 SV 514   SV 515                 50
          MV-408   (1) MDSLSVNQ----ILYPEVHLDSPIVTNKIVAILEYARVPHAYSLEDPTLC
  SV L 1-408 aa   (1) MDGQESSQNPSDILYPECHLNSPIVRGKIAQLHVLLDVNQPYRLKDDSII
       Consensus   (1) MD     Q    ILYPE HL SPIV  KI        V   Y L D

                        51                            SV 516             100
          MV-408  (47) QNIKHRLKNGFSNQMIINNVEVGNVIKSKLRSYPAHSHIPYPNCNQDLFN
  SV L 1-408 aa  (51) NITKHKIRNGGLSPRQIKIRSLGKALQRTIKDLDRYTFEPYPTYSQELLR
       Consensus  (51)    KH   NG      I     G                PYP   Q L

                        101                                               150
          MV-408  (97) IEDKESTRKIRELLKKGNSLYSKVSDKVFQCLRDTNSRLG--LGSELRED
  SV L 1-408 aa (101) LDIPEICDKIRSVFAVSDRLTRELSSGFQDLWLNIFKQLGNIEGREGYDP
       Consensus (101)     E   KIR        L    S             LG   G E

                        151                         SV 517                200
          MV-408 (145) IKEK--VINLGVYMHSSQWFEPFLFWFTVKTEMRSVIKSQTHTCHRRRHT
  SV L 1-408 aa (151) LQDIGTIPEITDKYSRNRWYRPFLTWFSIKYDMRWMQKTRPGGPLDTSNS
       Consensus (151)                   W  PFL WF  K  MR   K

                        201      SV 518                  SV 519           250
          MV-408 (193) PVFFTGSSVELLISRDLVAIISKESQHVYYLTFELVLMYCDVIEGRLMTE
  SV L 1-408 aa (201) HNLLECKSYTLVTYGDLVMILNKLTLTGYILTPELVLMYCDVVEGRWNMS
       Consensus (201)        S  L    DLV I  K     Y LT ELVLMYCDV EGR

                        251      SV 520                  SV 522           300
          MV-408 (243) TAMTIDARYTELLGRVRYMWKLIDGFFPALGNPTYQIVAMLEPLSLAYLQ
  SV L 1-408 aa (251) AAGHLDKKSIGITSKGEELWELVDSLFSSLGEEIYNVIALLEPLSLALIQ
       Consensus (251)  A   D             W L D  F  LG   Y   A LEPLSLA  Q

                        301                                        SV 522
          MV-408 (293) LRDITVELRGAFLNHCFTEIHDVLDQNGFSDEGTYHELIEALDYIFITDD
  SV L 1-408 aa (301) LNDPVIPLRGAFMRHVLTELQTVLTSRDVYTDAEADTIVESLLAIFHGTS
       Consensus (301) L D    LRGAF  H  TE   VL               E L  IF

                        351              *                                400
          MV-408 (343) IHLTGEIFSFFRSFGHPRLEAVTAAENVRKYMNQPKVIVYETLMKGHAIF
  SV L 1-408 aa (351) IDEKAEIFSFFRTFGHPSLEAVTAADKVRAHMYAQKAIKLKTLYECHAVF
       Consensus (351) I    EIFSFFR FGHP LEAVTAA  VR  M   K I   TL   HA F

                        401           416
          MV-408 (393) CGIIINGYRDRHGGSW
  SV L 1-408 aa (401) CTIIINGY--------
       Consensus (401) C IIINGY
```

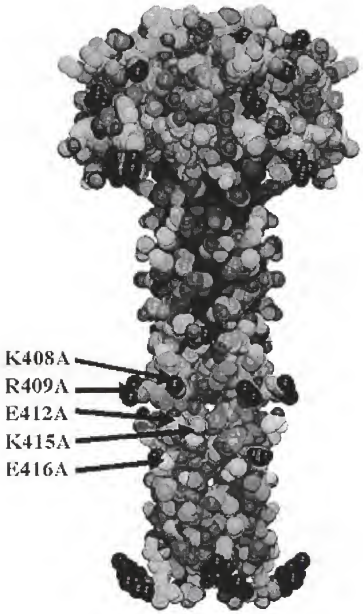
gstP protein. The only mutants capable of forming a detectable complex with the gstP protein are the SV 516 and SV 520 proteins and for both of these mutants we estimate approximately 50% of the total L expressed is detected in a complex with the gstP protein. These complexes appear close to the limit of detection for this particular assay and as such it is possible that some of the remaining mutants may be able to form a small amount of complex which goes undetected in the bead binding assay. Based on the levels of expression of the remaining mutants we estimate that the L proteins which complex at less than 20% of their total protein expression will go undetected in the bead binding assay. Evidence for this can be seen with the mutant SV 514 and SV 515 proteins which do not form a detectable complex with gstP in the bead binding assay but are able to form a barely detectable complex in the nucleocapsid binding assay (Fig. 17). Since the total amount of protein used in the nucleocapsid binding assay is approximately 9-10 fold more than the amount used in the bead binding assay it is the more sensitive assay. The bead binding assay seems to be a good test for whether or not a mutant is able to perform any RNA synthesis in that mutants which can form a detectable complex are able to give some RNA synthesis whereas mutant which can not form such a complex are unable to give detectable RNA synthesis (Table 6).

The polymerase complex of Sendai virus is composed of a homotetramer of the P protein and a monomer of the L protein. This complex through the P protein recognizes the encapsidated viral genome and performs both replication and transcription. This work provides evidence that the P and L proteins must interact in order to form a functional polymerase complex and that complex formation, while necessary, is not sufficient for RNA synthesis. Further it shows that the P protein binds to the amino-terminal 380 aa of both the Sendai and measles virus L proteins. Mutagenesis of several sites in each protein independently abolish binding to the P protein indicating that one site does not compensate for the others. These mutations did not disrupt the structure of most of the L proteins on the global scale because they retained binding to the gstC protein. In Sendai viruses two mutants, namely SV 516 and SV 520, were partially able to form a polymerase complex and they also gave partial RNA synthesis phenotypes which were much reduce when compared to the wt L protein. These data correlate with previous studies in which truncations and deletion analysis in several viruses indicated that the amino-terminal portion of the L protein binds to the P protein. In the SV-5 virus truncation and deletion analysis of the L protein indicated that the P binding site resides within the amino-terminal 1247 aa. Deletion analysis within

the first 1247 aa of the SV-5 L protein lead to a series of mutants which were all unable to bind to the P protein. Either the P protein binding site stretches across 1247 aa or deletion analysis is not the most effective method for finding the P binding site. Most likely deletions within this region of L lead to unstable proteins which can not properly fold and thus can not bind to the P protein. A similar situation occur in Sendai virus were carboxy-terminal truncations of L lost the ability to bind to the P protein at 895 aa. A single point mutation in SV L at S368R was unable to bind to the P protein (Chandrika et al., 1995a), which is just downstream of the SV 522 protein. The reciprocal change in MV 508 did not abolish binding. In measles virus the L protein could be truncated to the amino-terminal 408 aa further implicating this region as responsible for P protein binding.

Recently the crystal structure of the aa 321-432 containing the SV P protein oligomerization domain and a portion of the L binding site on P was published (Tarbouriech et al., 2000a). The structure of this domain of the P protein is a homotetrameric coiled coil structure (Fig. 22). This structure shows the location of two site-directed mutants (K408A / R409A and E412A / K415A / E416A) created in our lab which are deficient in leader RNA synthesis and transcription *in vitro* (Bowman et al., 1999).

Figure 22. A space filling model of the crystal structure of the Sendai virus P oligomerization domain with a portion of the L binding domain (Tarbouriech et al., 2000). The Sendai virus P protein forms a stable homotetrameric coiled coil structure. Mutations indicated by the arrows are site-directed mutations of the Sendai virus P protein which still bound to the L protein but abolished *in vitro* transcription (Bowman et al., 1999).

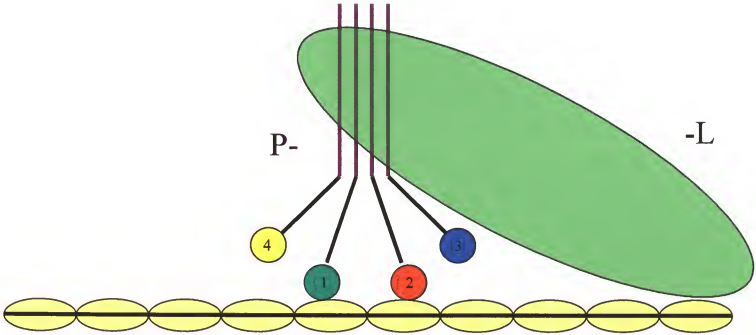


Tarbouriech, et al., 2000

These amino acids interact directly with the L subunit of the polymerase and while the mutations do not disrupt binding, they interfere with some aspect of viral transcription, possibly by promoting an incorrect conformation of the L protein. It is thought that the polymerase complex is composed of a P to L molar ratio of 4:1 (Tarbouriech et al., 2000b). Site-directed mutagenesis of the L binding domain of the P protein did not produced mutants which cannot bind L so individual groups of mutations do not disrupt the interaction at other sites. This suggests that this P domain is extremely robust as reflected in its crystal structure.

Previously a model for the P and L protein interaction was proposed but at the time it was thought that P and L were in a 3:1 molar ratio. The model we propose for the P-L interaction is an update to this previous model in that the L protein via the amino-terminus wraps around the coiled coil structure of the P protein analogous to a hand around a screwdriver (Fig. 23). Downstream of this P and L interaction the P protein is able to interact with the nucleocapsids and thus bring the polymerase complex to the template, denoted by globular numbered heads. One NP polypeptide interacts with 6 nucleotides of the template (Calain and Roux, 1993). At any one time perhaps only two of the P proteins, denoted 1 and 2, may interact directly

Figure 23. A model for the Sendai virus P-L interaction and for RNA synthesis. The model shows the P protein interacting with the nucleocapsid template via two of the four possible P-template binding domains. The portion of the P protein in purple indicates the P oligomerization domain and portions of the L binding domain (aa 321-432) for which a crystal structure is available (Tarbouriech N, et al., 2000). The nucleocapsid binding domain and remaining portion of the L binding domain of the P protein are indicated by black lines below for which no structural information is available. Black lines above indicate the amino-terminus of the protein (aa 1-320) for which no structural information is available. The numbered colored circle indicate region of direct contact between the P protein and the nucleocapsid template. The amino-terminal portion of the L protein is indicated by the cylinder which encircles the P protein.



Nucleocapsid Template

with the two NP molecules (Fig. 23). For the polymerase complex to move down the template the L protein may rotate the P protein thus breaking one of the P-template interaction, denoted as 1. The remaining P-template interaction allows the complex to swing around contact 2 allowing another P protein to form a new interaction at contact 3 (Fig. 23). This motion would move the polymerase complex down one NP protein on the template allowing it to transcribe or replicate the next set of 6 nucleotides.

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BIOGRAPHICAL SKETCH

David Edward Holmes was born June 12th, 1969, in Philadelphia, Pennsylvania. He attended school in Philadelphia until 9th grade when he moved with his family to Port Saint Lucie, Florida, in 1984. He graduated from Fort Pierce High School in 1987. In 1987 he went to the University of Florida to major in aerospace engineering and after a brief hiatus from school switched majors and graduated in 1996 with a Bachelor of Science in microbiology and cell science. Immediately after graduation he began graduate school at the University of Florida in the laboratory of Dr. Sue Moyer. After receiving his Doctor of Philosophy, David, his wife, Marcela, and his daughter, Daniela, will move to Laramie, Wyoming, where he will begin his post doctoral studies on blue tongue virus with the United States Department of Agriculture. Eventually he would like to pursue a career in academia so that he may remain active in research and teaching. Currently he and his wife, Marcela, are awaiting the birth of their second child, he or she is due mid-November.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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